

ELECTRON MICROSCOPY OF THE HUMAN PLACENTA¹

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TWENTY FIGURES

It has become apparent in the last decade that the placental membranes in man and other mammals, which mediate the metabolic exchange between the maternal and fetal blood streams, are exceedingly complex in their cytological and histochemical structure (cf. Amoroso, '52; Wislocki, '55). The human placenta is of the hemochorial type according to the classification of Grosser. The trophoblast, which constitutes the principal component of the placental membrane, consists of a cellular layer (the Langhans cells) and a syncytial layer (the syncytium) which clothe the chorionic or placental villi. The trophoblast, the basement membrane upon which it rests, and the contiguous fetal capillaries form the placental barrier, through which physiological exchange occurs between mother and fetus and *vice versa*. Numerous organelles and a variety of proteins, enzymes, carbohydrates and lipids have been demonstrated by cytological and histochemical methods in the cytoplasm of the syncytial trophoblast (Wislocki and Bennett, '43; Dempsey and Wislocki, '44, '45; Wislocki, '55). These cytoplasmic structures and substances appear to play important, although far from completely understood, rôles in the regulation of placental exchange of various metabolites, in the synthesis of placental hormones and fetal proteins, and in the intrinsic growth and maintenance of the placenta itself (cf. Wislocki, '55).

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In addition to the placental labyrinth which is composed of branching villi, there are peripheral cellular masses termed the trophoblastic cell columns and trophoblastic shell which are collectively called the peripheral cytotrophoblast. The cells composing these differ cytologically from the Langhans cells and the syncytium clothing the placental villi (Wislocki, '51, '55).

With this body of information available concerning the human placenta, it has been of interest with the advent of the electron microscope to investigate the fine structure of this organ and to compare and correlate the findings with the existing cytological and histochemical data. The present paper gives an account of the human placenta investigated with the electron microscope, and discusses the observations in terms of previous knowledge. Some of the findings have already been briefly presented (Dempsey and Wislocki, '53). Less complete observations than the present ones have also been published by Boyd and Hughes ('54), a discussion of which will be deferred until after the presentation of our own results.

MATERIAL AND METHODS

The material consisted of two human placentas removed by hysterectomy for therapeutic reasons at 9 and 10 weeks of gestation. The specimens were turned over to us for use through the interest and courtesy of Dr. Duncan E. Reid of the Boston Lying-In Hospital, to whom we wish to express our appreciation. The material also included specimens of two additional human placentas at full term. Blocks of tissue were fixed within 15 minutes to half an hour after the specimens were obtained. From the hysterectomy specimens, pieces of tissue were fixed separately from the placental labyrinth, from the trophoblastic cell columns and trophoblastic shell and from the decidua basalis and decidua vera. From the placentas at full term, samples were taken solely of chorionic villi from the placental labyrinth.

Small bits of these tissues were placed in fixative, consisting of 1% osmic acid buffered with Ringer's solution and

veronal to pH 7.5. These specimens were fixed for 4 hours at room temperature, washed briefly in three changes of distilled water and dehydrated in 70%, 95% and absolute ethanol. They were then infiltrated with methacrylate monomer (three parts butyl methacrylate and one part methyl methacrylate) for 3 hours, in three changes of the plastic mixture. Finally, they were transferred to no. 3 gelatin capsules which were filled with the plastic monomer to which benzoyl peroxide had been added as a catalyst (13.5 mg catalyst per milliliter plastic). The plastic was allowed to set slowly overnight at room temperature, after which polymerization was completed in 24 hours in an incubator at 45°C.

After embedding, the gelatin capsules were removed by soaking in warm water. The glassy-hard blocks were trimmed for sectioning under a low-power binocular dissecting microscope. They were then placed in the chuck of a modified rotary microtome (Geren and McCulloch, '51; Dempsey and Lansing, '53) and sections were cut at thicknesses of .025 to 0.1 μ . The thicker sections proved best for cellular topography whereas the thinner sections permitted the best resolution of fine detail, such as the double layer of the nuclear membrane and the detailed internal structure of mitochondria. The sections were floated and spread upon a 30% acetone-solution, lifted upon collodion-coated, copper-mesh grids, dried and examined in an RCA electron microscope, Model EMU, at initial magnifications of 1000 to 6000 diameters. The photographic negatives exposed at these magnifications were subsequently enlarged as desired.

For purposes of comparison with ordinary histological reactions pieces of the placentas were fixed in Zenker's acetic acid fluid, Orth's fluid and Heidenhain's susa fluid. These tissues were embedded in paraffin and 5 μ sections were cut. These were deparaffinized, and stained by eosin and methylene blue, Gomori's chrome alum-hematoxylin and phloxine, Masson's trichrome mixture and McManus' periodic acid-Schiff procedure.

OBSERVATIONS

There are no essential differences apparent between the two human placentas of 9 and 10 weeks of gestation, so that a combined description of them should suffice. Moreover, the structure of the placenta seems so clearly shown in the electron micrographs that it is deemed superfluous to accompany them by lengthy descriptions. The features of the chorionic villi are shown in figures 1 to 7, those of the peripheral trophoblast (cell columns and trophoblastic shell) in figures 8 to 10, those of the decidua basalis and decidua vera in figures 11 to 15 and the structure of the chorionic villi at full term in figures 16 to 20.

The chorionic villi. In the two specimens of two months of gestation, the trophoblast covering the chorionic villi consists of an outer syncytial layer and an inner layer of Langhans cells (figs. 1, 2). The free surface of the syncytium bordering the intervillous space is covered by a profusion of microvilli of varying appearances (figs. 1, 2, 4, 5, 6). Often they are fairly long and slender and possess enlarged or bulbous tips. However, in many places they are shorter and thicker, while in still other places they are quite flat and few in number. In occasional places, small promontories of cytoplasm studded by microvilli protrude into the intervillous space (fig. 5). Larger tabs of syncytium containing several or many nuclei are encountered here and there and the surface of these is also variously provided with microvilli (fig. 6). The microvilli have a dark contour and when they are especially large and bulbous the cytoplasm within them shows a greyish stippling.

Beneath the microvilli the broad expanse of the syncytial cytoplasm extends down to the subjacent Langhans cells, or between them to the basement membrane upon which the trophoblast rests. The syncytium contains small, irregularly shaped, ovoid nuclei which exhibit considerable electron density. The syncytial cytoplasm is completely filled by a variety of bodies. The most conspicuous are, perhaps, moderately large, round or oval clear vesicles of rather uniform

size occupying much of the cytoplasm (figs. 1, 2, 4), but most numerous in the inner two-thirds of the syncytium. Scattered between the clear vesicles are numerous, slightly smaller, dark objects recognizable as mitochondria (fig. 3). Also present are larger, more infrequent, spherical objects possessing somewhat irregular outlines, which are identifiable as fat droplets (figs. 2, 3). In some regions, in a zone just beneath the microvilli, there are variable numbers of quite large vacuoles containing finely stippled or flocculent material in their lumens (figs. 1, 2, arrows). These vacuoles are as a rule larger than the ovoid vesicles of more uniform size scattered throughout the syncytium. The latter usually have a well-defined, confining membrane which is stippled, beaded or granular, whereas each of the large vacuoles is surrounded by quite an indistinct membrane. The large vesicles occur occasionally within tongues of cytoplasm protruding from the surface of the syncytium (fig. 5) but are encountered most frequently in the marginal cytoplasmic zone of the syncytium (fig. 2, arrows). Furthermore, between the bases of the microvilli, there are occasional bays and invaginations of the surface cytoplasm which, in conjunction with the subjacent vesicles, suggest that the latter may contain plasma entrapped or engulfed by the motile, unstable cytoplasmic border of the syncytium. These large, marginal vacuoles would appear to be unrelated to the much more numerous, smaller, clear vesicles filling much of the syncytium which are enclosed by a well-defined membrane. In the interstices between the mitochondria, vesicles and fat droplets in the syncytium, greyish flocculent material is apparent.

The Langhans cells stand out conspicuously (figs. 1, 2, 4). Both their cytoplasm and large nuclei are less dense than those of the syncytium. The most evident element of their cytoplasm is the mitochondria, which are relatively few in number but considerably larger than those in the syncytium (fig. 3). Besides the mitochondria, their cytoplasm contains a few vesicles with granular or beaded outlines, similar to the numerous ones present in the syncytium. The cytoplasm

also contains occasional structures recognizable as typical ergastoplasm, or ergastoplasmic membranes and sacs, as described and defined by Weiss ('53). In the actual electron micrographs as seen in one plane only, these appear as double membranes or threads with granular outlines. Some of them appear to be vesicular at one end, apparently representing transition forms between ergastoplasmic membranes and vesicles. The ergastoplasmic membranes of Weiss are the equivalent of the so-called "endoplasmic reticulum" of Porter ('53), both representing the basophilic cytoplasmic substance (ribonucleoprotein) of light microscopy. Aside from the several formed elements the cytoplasm possesses much relatively clear plasma or matrix containing a modicum of stippled or flocculent material.

The cytoplasm of the Langhans cells appears to be enveloped by a very delicate plasma membrane. The Langhans cells are apposed on their outer surfaces to the syncytium and on their basal surfaces to the basement membrane of the stroma of the chorionic villi. The plasma membrane and basal cytoplasm of the syncytium, apposed to the delicate plasma membranes of the Langhans cells, are quite dense in character. Between the plasma membranes of the syncytium and Langhans cells, small open clefts are sometimes apparent. Basally, the Langhans cells rest upon a narrow, continuous basement membrane of nearly homogeneous grey appearance to which wisps of collagen of the open-textured stroma of the villus attach themselves.

Laterally, many of the Langhans cells abut one another, but the cell boundaries are inconspicuous, because the plasma membranes seem to be so thin and delicate. Here and there, where narrow clefts occur between two contiguous Langhans cells, separate plasma membranes are apparent. In other places, the cytoplasm of the dark-appearing, basal portion of the syncytium dips down between the Langhans cells to abut directly upon the basement membrane.

The stroma (figs. 1, 7) exhibits well-defined mesenchymal fibroblasts scattered in a loose-textured matrix containing a

delicate network of collagenous fibers. The mesenchymal cells contain mitochondria and a moderate amount of ergastoplasm of characteristic appearance. Occasional Hofbauer cells are visible. These are characterized by more rounded contours than the mesenchymal cells, the absence of ergastoplasm and the presence of a number of large vacuoles of variable sizes with ill-defined walls. Fetal capillaries are lodged in the stroma of the villus (fig. 7); they possess endothelial cells which are well provided with cytoplasm, and rest upon a delicate basement membrane.

The peripheral cytotrophoblast (cytotrophoblastic cell columns and shell). The peripheral trophoblastic cells (figs. 8, 9, 10), comprising the cell columns and trophoblastic shell, differ in morphology from the Langhans cytotrophoblasts of the chorionic villi. Their nuclei are similar but their cytoplasm differs.

There are essentially two varieties of peripheral cytotrophoblasts depending upon the relative amount and pattern of cytoplasmic glycogen and ergastoplasm which they contain. The first exhibits extensive grey, cytoplasmic fields of glycogen (fig. 8), the mottled appearance of which is caused by branching chains of electron dense substance. Mitochondria are variously scattered throughout the cytoplasm and the fields of glycogen are interrupted and subdivided by masses of ergastoplasmic and cytoplasmic material. The latter are located mainly in the neighborhood of the nucleus and the cell membrane with occasional strands connecting the principal masses.

The second variety of peripheral trophoblasts comprises cells in which glycogen is not so abundant and ergastoplasmic membranes are more uniformly dispersed throughout the cytoplasm (fig. 9). All degrees of transition between the two forms are apparent.

Between the trophoblastic cells there is a variable amount of interstitial substance; between some cells this material is slight in amount, but between others it is abundant. The substance is either granular or forms irregularly shaped

masses of varying size, both exhibiting considerable electron density.

The decidua. No effort was made to study the different layers of the decidua. Examination was limited merely to the identification of typical decidual cells and uterine glandular epithelium in the decidua basalis and vera.

In both localities the cells are similar. Figures 11, 12, 13 and 15 illustrate the appearances of typical large decidual cells. Their cytoplasm contains mitochondria of varying size, membranous structures (ergastoplasm) and uniformly stippled material (glycogen) (figs. 11, 12). Many of the cells contain fat droplets (fig. 11). Some of the cells are approximately spherical in shape, others have complicated outlines, and still others are fusiform. In the superficial layer of the decidua vera the cells are generally more loosely arranged and fusiform in shape (fig. 12). An interstitial matrix consisting of a network of collagenous fibers and fibrils, and flocculent ground substance surrounds the cells. The appearance of some of the fusiform decidual cells suggests that collagenous fibrils are formed within their cytoplasm and released at the tips of the extended cell processes into the surrounding matrix (fig. 12).

The epithelium of the uterine decidual glands is of interest, in that the cells possess numerous, short microvilli on their luminal surfaces (fig. 14).

The chorionic villi at full term (figs. 16, 17, 18, 19, 20). The villi at full term are covered by syncytium which is much thinner than at 10 weeks of gestation but which still bears microvilli. The processes on the cells are, however, much shorter than they were previously. Although the cytoplasm still contains some mitochondria and lipid droplets, it no longer shows the profusion of small vesicles of the earlier period. Residual ergastoplasm is recognizable in the cytoplasm surrounding the nuclei which are gathered in groups between adjacent dilated capillaries. Some of the residual ergastoplasmic vesicles appear to be extremely dilated. In addition, some regions of the syncytium contain larger vacu-

oles or vesicles, many or all of which may be artifacts or the result of degenerative changes. It is uncertain whether all of these are to be traced to former ergastoplasmic vesicles. The basement membrane upon which the trophoblast rests is much stouter than at the earlier period and the collagenous fibers are coarser than before. Thin-walled vessels of sinusoidal capillary dimensions indent the trophoblast (figs. 16, 18, 19); the walls of these vessels are formed of endothelial cells resting upon a basement membrane. Between the basement membranes of the trophoblast and capillary, there is a narrow space in which collagenous fibers are apparent (fig. 16).

In many fields it is evident that large, clear, somewhat flattened cells are present in the trophoblast. These are basally placed beneath the syncytium, but are always separated from the stroma of the villus by the basement membrane upon which they rest. These cells appear to represent residual Langhans cells which have persisted until full term (figs. 19, 20).

DISCUSSION

It is of interest to examine to what degree the findings with the electron microscope coincide with, or differ from, the cytological and histochemical observations made with the light microscope.

The young chorionic villi. The correspondence of the lipid droplets, mitochondria and brush border of the syncytium in the light microscope, to the equivalent structures in the electron microphotographs is at once apparent. The microvilli of the electron photographs show considerable variability with respect to their length, shape and number, resembling the variable cytology of the brush border previously described (Wislocki and Bennett, '43).

The large vesicles or vacuoles seen in variable numbers in the surface zone of the syncytium and their probable formation by engulfment of plasma coincide with the vesicles described by Wislocki and Streeter ('38) and Wislocki and Bennett ('43), the formation of which they ascribed to the

process of pinocytosis. The evidence for this interpretation rested upon the variable morphology of the surface of the syncytium which suggested that it was plastic and motile (Wislocki and Bennett, 1.c), as well as upon observations derived from tissue cultures in which syncytial trophoblast was observed to be actively motile (Jones et al., '43).

The numerous vesicles of rather uniform size and granular outlines seen in the syncytium with the electron microscope, we regard as ergastoplasm (Weiss, '53), or endoplasmic reticulum (Porter, '53), and as equivalent to the cytoplasmic basophilia of the syncytium observed with the light microscope. As Dempsey and Wislocki ('45) have shown, the latter is attributable to ribonucleoprotein. We equate these vesicles with cytoplasmic basophilia for several reasons. The ergastoplasm of various kinds of cells observed with the electron microscope consists of membranous sacs exhibiting a slit-like or dilated lumen which is bounded by a thin, moderately dense membrane the outer surface of which is associated with a variable number of small electron-dense granules so that it presents a stippled or beaded appearance (Weiss, '53). The vesicles of the syncytium have this beaded appearance. Moreover, they correspond fairly well in their distribution and relative numbers to the observed distribution of cytoplasmic basophilia (Dempsey and Wislocki, '45; Singer and Wislocki, '48). The beaded vesicles and the basophilia are characteristic of the syncytium in the first trimester of pregnancy, whereas they have nearly disappeared at full term. The various morphological appearances of the ergastoplasm in different cell types are probably related to various physiological states and the rate of protein synthesis. The vesicular and distended saccular types of ergastoplasm are probably indicative of greater activity than the collapsed saccular or membranous forms. In the placenta the ergastoplasm of the syncytial trophoblast is completely vesicular, whereas that of the mesenchymal cells in the stroma of the villi is of the membranous, saccular kind. Wislocki, Dempsey and Fawcett ('48) have postulated that the basophilic substance (ribo-

nucleoprotein) of the syncytium synthesizes the fetal plasma proteins until such time as the fetal liver takes over that function. Certainly, as gestation advances, cytoplasmic basophilia of the syncytium diminishes steadily.

It is worthy of note that the vesicles observed with the electron microscope should be large enough to be seen with the highest power of the light microscope. With reference to this, it is evident that after some fixatives, including Altmann's fluid which contains osmic acid and is the fixative of choice for electron microscopy, numerous vesicles of corresponding shape and number are faintly recognizable with the light microscope (Wislocki and Bennett, '43, figs. 2, 13, 14). However, with a fixative such as Zenker's fluid, recommended for the demonstration of cytoplasmic basophilia, the pattern of the basophilia is uniformly stippled rather than vesicular (Dempsey and Wislocki, '45, fig. 2). This raises the question which cannot be answered of the degree to which the different patterns of the basophilia and ergastoplasm seen with the light and electron microscopes respectively, correspond to the actual condition of the nucleoprotein in the living cytoplasm.

The suggestion is offered that the relative density of the cytoplasmic ground substance of the syncytium in the electron microscope may be due to the placental transfer of iron, since a rather intense Prussian blue reaction for iron is characteristic of the syncytium (Dempsey and Wislocki, '44). Areas of particular electron density, present in the endometrial glands, glandular secretion and chorionic areolae of the sow's placenta, have been interpreted as being due to the placental transmission of iron (Dempsey, Wislocki and Amoroso, '55).

The peripheral trophoblast. Two of the major cytoplasmic components of the trophoblast seen with the light microscope, namely glycogen and cytoplasmic basophilia (Dempsey and Wislocki, '44, '45), are typically present in the electron micrographs. The glycogen forms delicately flocculent, cytoplasmic fields, whereas the cytoplasmic basophilia is present as convoluted masses or strands of ergastoplasmic membranes.

As in light microscopy no collagenous fibers appear to be present in the ground substance between the trophoblasts. However, the ground substance contains material in masses and strands which exhibits considerable electron density, suggesting the presence of iron, a finding which is consonant with the strong histochemical reaction for iron observed there (Dempsey and Wislocki, '44). In our electron micrographs there is no evidence which suggests that, like mesenchymal cells and decidual cells, either the peripheral trophoblasts or the Langhans cells are producing collagenous fibrils.

The decidua. The presence of mitochondria, glycogen, fat droplets, and some ergastoplasm in the typical large decidual cells coincides with the findings of light microscopy (Wislocki and Dempsey, '48). The seeming production of collagen fibrils by the decidual cells is a feature detected only by the electron microscope; with respect to the production of collagen the decidual cells differ from the cytotrophoblasts.

The presence of microvilli upon the free surface of the decidual glandular epithelium is also a finding which was unsuspected on the basis of light microscopy. The microvilli of the syncytium of the chorionic villi, one might postulate, should be related principally to the process of absorption of material by the trophoblast from the maternal blood plasma. On the other hand, the microvilli on the endometrial glandular epithelium, one might presume, would be involved in the process of secretion. Thus, apparently, microvilli do not necessarily bespeak either the one process or the other; instead, they would appear, as the case may be, to increase either the effective absorbing or secretory surface. In the pig's placenta, too, microvilli appear on the surfaces of secretory glandular cells as well as upon seemingly absorptive chorionic cells (Dempsey et al., '55).

The chorionic villi at full term. Wislocki and Bennett ('43) called attention to the stubble-like appearance of the surface of the syncytium of the chorionic villi at full term, relating it to the brush border of earlier periods. In keeping with their observation, the electron microscope reveals that the

syncytium at full term is covered with short, irregular microvilli.

The thinnest areas of the placental membrane at term are revealed by the electron microscope, as well as by light microscopy, to consist of a thin lamina of syncytium which rests on a stout basement membrane, a connective tissue space containing some collagenous fibers, and the wall of a sinusoidal capillary composed of an additional basement membrane lined internally by endothelium.

Although the thinnest areas of the syncytium at full term had previously been designated as "epithelial plates," and likened structurally to Bowman's capsule of renal glomeruli or to the alveolar membrane of the lungs (Bremer, '16), electron microscopy of renal glomeruli (Dempsey and Wislocki, '55) and of pulmonary alveoli (Low, '53) reveals that there is actually very little structural similarity between these various membranes. Only the placental membrane, to mention a major difference, bears microvilli. Thus the postulation that the thinnest parts, or "epithelial plates," of the placenta must be excretory areas, because of their similarity to renal glomeruli or to the respiratory membrane, has no convincing morphological basis.

Another point of interest brought out by electron microscopy is that the trophoblast at full term contains numerous, flattened, basally located, residual Langhans cells. Contrary to general belief with daylight microscopy, Wislocki and Bennett ('43, figs. 17, 18) maintained that some residual Langhans cells appeared to be present in the chorionic villi at full term; they described basally placed clear cells resting upon the basement membrane without any argyrophil reticulum separating them from the syncytium. Examination of the chorionic villi at full term with the electron microscope adds substantial proof of their surmise, by demonstrating numerous basally situated cells, distinguishable from the syncytium by their clearer cytoplasm and larger mitochondria but also clearly separated from the stroma of the villus by the stout basement membrane upon which the trophoblast rests. Fur-

thermore, the electron micrographs of both the early and late placental specimens provide additional evidence that the Langhans cells are not separated from the syncytium, or encapsulated by a basement membrane, collagen or any other interstitial material. This has previously been a moot point (cf. Wislocki and Bennett, '43).

The findings reported here substantiate and elaborate a brief previous report (Dempsey and Wislocki, '53). Meanwhile, a paper by Boyd and Hughes ('54) has appeared, upon the electron microscopy of the chorionic villi of a human embryo of 6 mm crown-rump length. This specimen is much younger than our two embryos which were close to 3 cm in length. However, Boyd and Hughes limited their examination solely to the chorionic villi of their specimen. Their findings are in agreement with ours with regard to the varied character of the microvilli and the presence of mitochondria and lipid droplets. In addition, they discerned densely packed vesicles about $0.3\ \mu$ in diameter as well as some much larger vacuoles and suggested that the larger ones might be formed by dilatation of the smaller ones. Furthermore, the presence of many vacuoles beneath the syncytial surface suggested to them that they might be the result of absorption, possibly by pinocytosis.

In regard to vesicular structures their account differs from ours in that we have distinguished two distinct kinds, namely: (1) numerous, widely dispersed small vesicles of relatively uniform size with granular outlines, which we have equated with cytoplasmic basophilia due to the presence of ribonucleoprotein, and (2) more irregular-sized vacuoles with indistinct walls situated usually close to the surface of the syncytium, which we believe to be formed as the result of absorption of maternal blood plasma by pinocytosis. As a result of previous acquaintance with the cytology of the trophoblast at different ages, we are of the opinion that absorption by pinocytosis characterizes quite young embryos to a greater degree than older ones, as a consequence of which the much younger specimen of Boyd and Hughes appears to exhibit

more pinocytosis and relatively less cytoplasmic basophilia than our somewhat older specimens. It should be pointed out, however, that their electron micrographs, as reproduced in their article, appear to possess less definition than ours, thus rendering the possible distinction of two kinds of vacuoles difficult.

SUMMARY

The human placenta at 10 weeks of gestation and at full term has been studied with the electron microscope and the findings compared with known placental cytology and histochemistry.

Electron micrographs of the chorionic villi at 10 weeks of gestation reveal abundant microvilli on the surface of the trophoblastic syncytium and numerous mitochondria and uniform-sized vesicles with granular outlines in the interior of the syncytium. The vesicles are regarded as representing a form of ergastoplasm equivalent to cytoplasmic basophilia (ribonucleoprotein) of the ordinary light microscope. The superficial portion of the syncytium contains variable numbers of larger vacuoles of uneven size and indistinct contours; these are believed to represent fluid trapped and engulfed by the motile, unstable surface of the syncytium as a result of the process of pinocytosis.

In the peripheral trophoblasts of the trophoblastic cell columns and shell, glycogen, mitochondria and ergastoplasm are recognizable, the latter being equivalent to the cytoplasmic basophilia (ribonucleoprotein) seen with the light microscope. Material of relatively high electron density located interstitially between the peripheral trophoblasts is interpreted as representing iron, known to be abundant there.

The chorionic villi at full term are covered by syncytium which bears short microvilli on its free surface. The placental barrier, even in the thinnest places where sinusoidal capillaries indent the syncytium, consists of a layer of syncytium bearing microvilli, a stout basement membrane, a connective tissue space containing collagen fibrils, and the wall of a capillary consisting of a basement membrane lined internally

by endothelium. Even in the thinnest places the placental barrier shows no structural similarity to either Bowman's capsule of renal glomeruli or to the alveolar membrane of the lungs. A considerable number of flattened residual Langhans cells are revealed by the electron microscope in the trophoblast at full term.

The ultrastructure of the decidual cells is illustrated. The epithelial cells of the decidual uterine glands possess short microvilli on their surfaces.

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DESCRIPTION OF PLATES

Figures 1 to 15 inclusive illustrate a placenta of 9 weeks of gestation, while figures 16 to 20 represent the placenta at full term.

PLATE 1

EXPLANATION OF FIGURE

1 Section through the edge of a chorionic villus at 9 weeks of gestation. The clear area at the upper left is the maternal intervillous space. Running diagonally across the lower right is the basement membrane of the trophoblast, beneath which is fetal connective tissue. Portions of three Langhans cells (LC) lie just above the basement membrane. These cells have large mitochondria (M) a few ergastoplasmic structures and cytoplasm containing sparsely distributed granules. The cell membranes are delicate, with wavy contours and occasional interdigitations with the plasma membranes of contiguous elements. The syncytial trophoblast lies between the Langhans cells and the intervillous space. Its free border is irregular, exhibiting bays, promontories and many slender microvilli with slightly bulbous terminations. Occasional fat droplets (black in the micrograph) have been distorted in sectioning. At the arrow a large vacuole is seen. This is one of the large apical vacuoles, also illustrated in figures 2 and 4 which we interpret as a product of pinocytosis. Besides these, numerous smaller, spherical vesicles, bounded by a distinct granular membrane, occur in the middle and basal portions of the syncytium. These have the distribution of the cytoplasmic basophilia, observed with the daylight microscope, and presumably represent dilated ergastoplasmic sacs. $\times 10,000$.

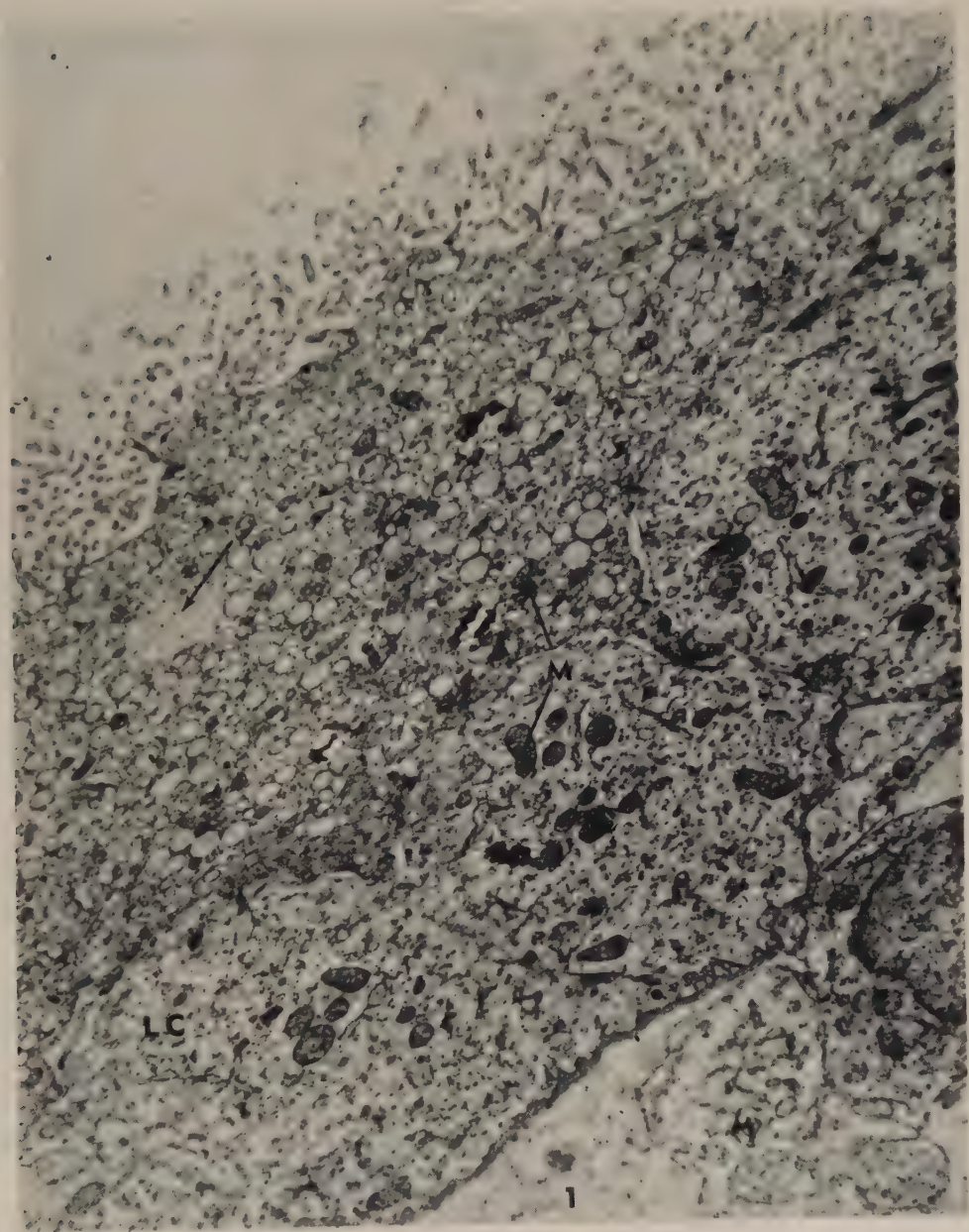


PLATE 2

EXPLANATION OF FIGURE

2 Section through the wall of a villus, showing profuse and elongated microvilli projecting into the intervillous space. Several large vacuoles are present in the apical syncytium (arrows). The nuclei of two Langhans cells and a portion of a nucleus of the syncytium are visible. This section is somewhat thicker than that illustrated in figure 1. $\times 8,000$.

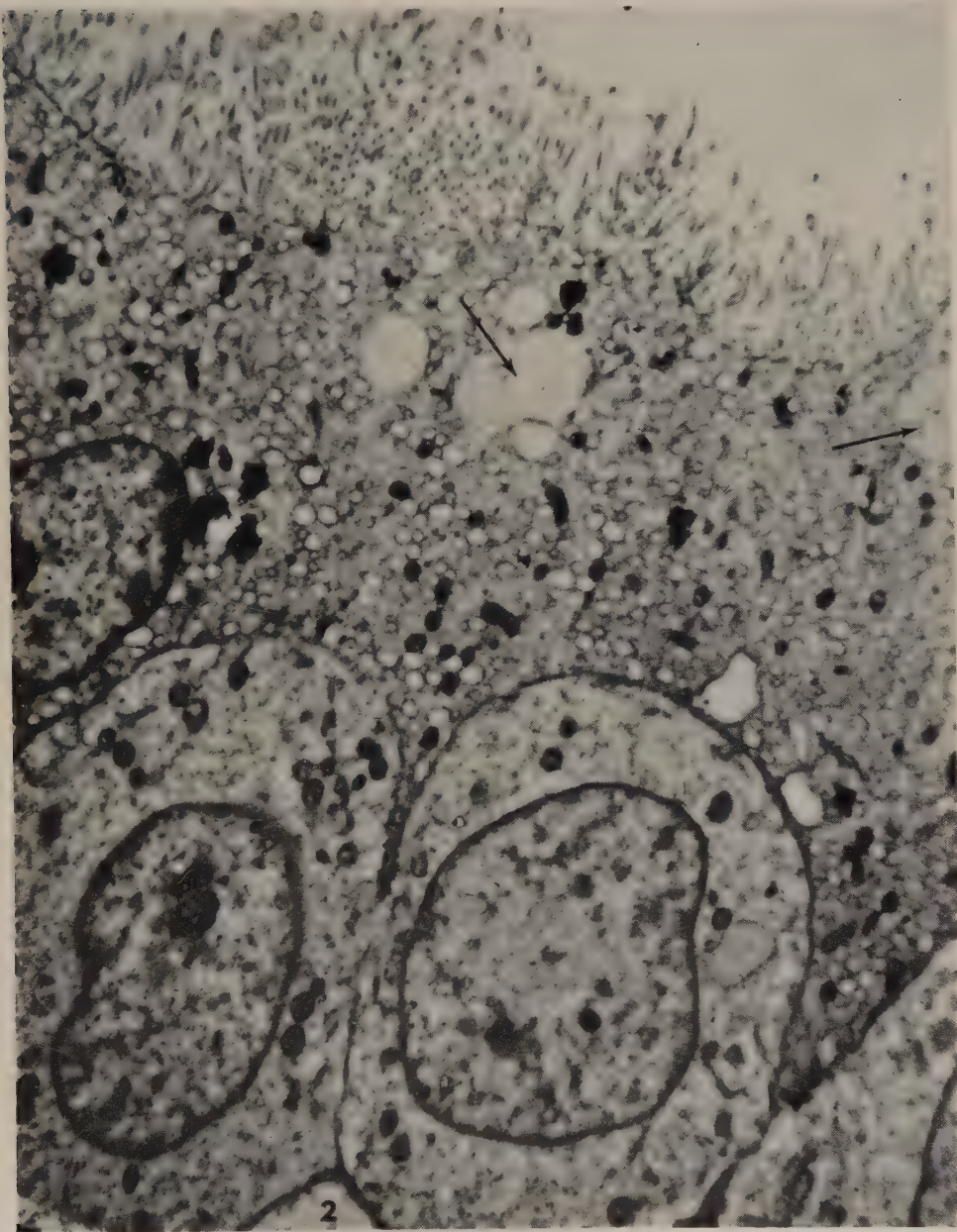


PLATE 3

EXPLANATION OF FIGURES

3 Section through a portion of a Langhans cell and the overlying syncytium. An edge of a nucleus (N) in the syncytium appears above, and the Langhans nucleus (N) below. The figure illustrates the differences between the mitochondria (M) of the two regions. In addition, a dark fat droplet and some dilated ergastoplasmic sacs appear in the syncytium. $\times 15,000$.

4 Another section through a villus, illustrating a variation in the appearance of the surface microvilli. Mitochondria, ergastoplasm and granular cytoplasm are also apparent. $\times 8,000$.

5 An area from the surface of the syncytium, exhibiting several of the large apical vacuoles including one within a promontory which projects into the intervillous space. $\times 16,000$.

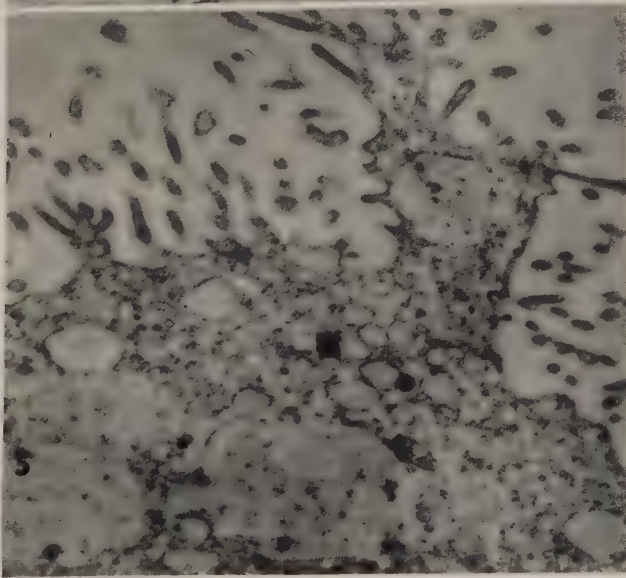
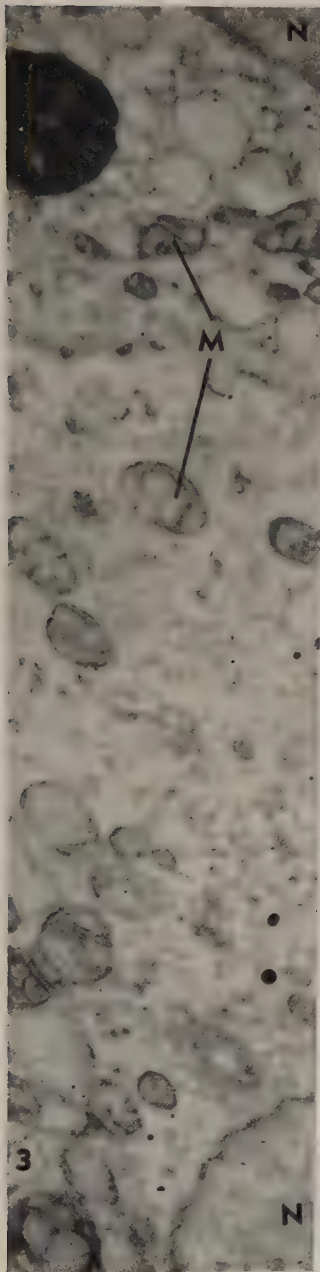


PLATE 4

EXPLANATION OF FIGURES

6 Section through a tab of syncytium. The variable character of the syncytial border is illustrated; it is comparatively smooth on the left but irregular on the right. Three nuclei are visible. The large number of ergastoplasmic sacs is in good accord with the marked basophilia of such regions as revealed with the daylight microscope both phenomena indicate the presence of cytoplasmic ribonucleoprotein. $\times 8,000$.

7 Section through a capillary in the stroma of a villus. Portions of 4 erythrocytes are visible within the capillary. Above and beneath the capillary are portions of two mesenchymal cells. The ergastoplasmic sacs of the cells (arrows) are irregular in shape, somewhat dilated, and filled with an amorphous substance of moderate density. $\times 8,000$.

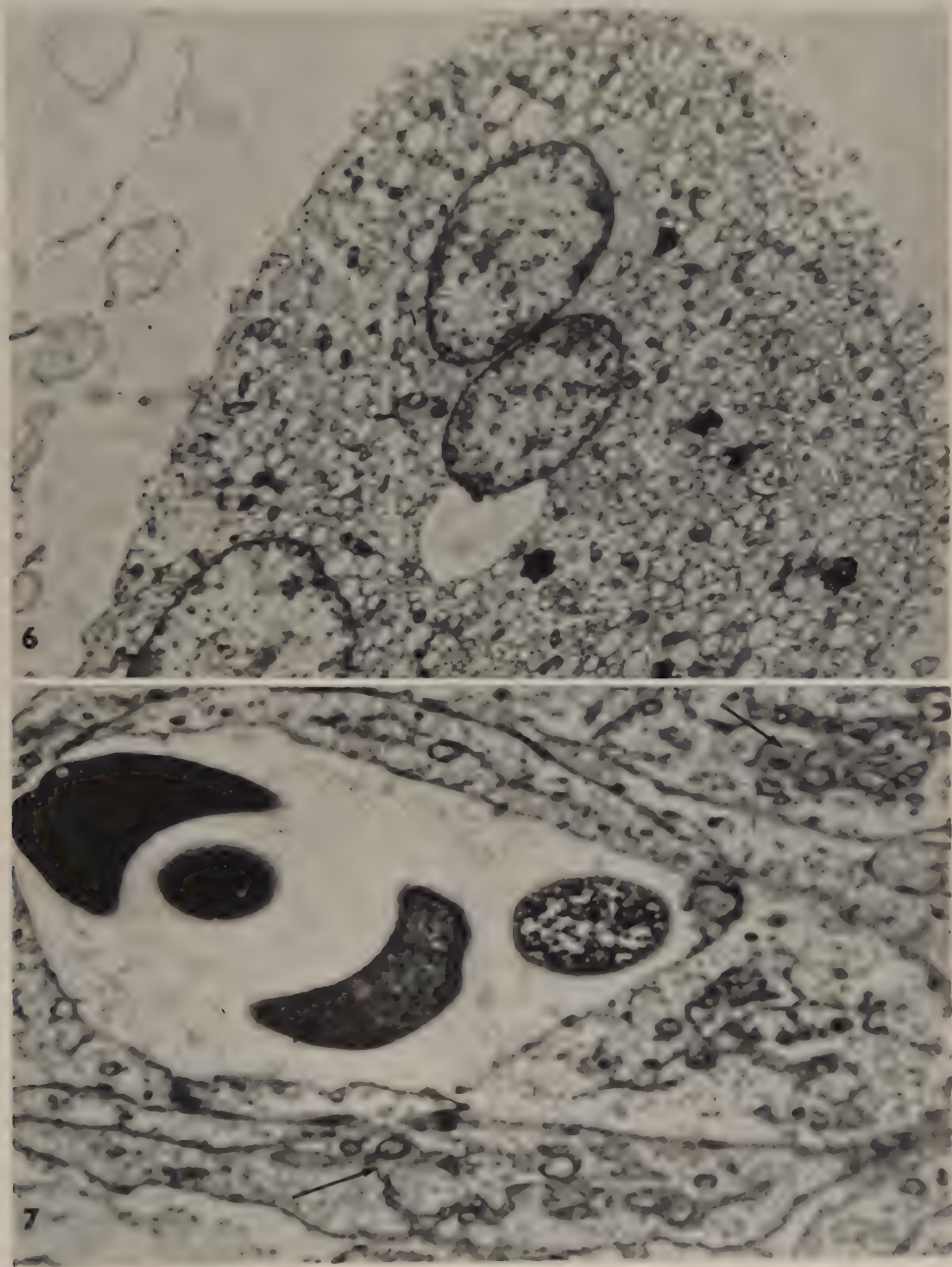


PLATE 5

EXPLANATION OF FIGURES

8 A cytotrophoblast from a cell column. The cytoplasm contains large amounts of glycogen which appear as grey areas. Scattered through these fields are strands and small masses of darker substances. These consist of ergastoplasmic structures, mitochondria and occasional lipid droplets. The ergastoplasm corresponds in amount and distribution to cytoplasmic basophilia seen with the light microscope. $\times 8,000$.

9 A trophoblastic cell in which glycogen is scanty, with ergastoplasmic sacs, mitochondria and granular cytoplasm widely dispersed throughout the cytoplasm. A band of interstitial substance containing masses and granules of electron-dense material is seen on the right hand side of the photograph. $\times 15,000$.

10 A trophoblastic cell near the edge of a cell column. Between it and the intervillous space, shown in the upper right corner, is a dark layer of syncytium containing many ergastoplasmic vesicles, mitochondria and several larger lipid droplets. The cytotrophoblast is of the variety having little glycogen and evenly dispersed organelles. $\times 8,000$.

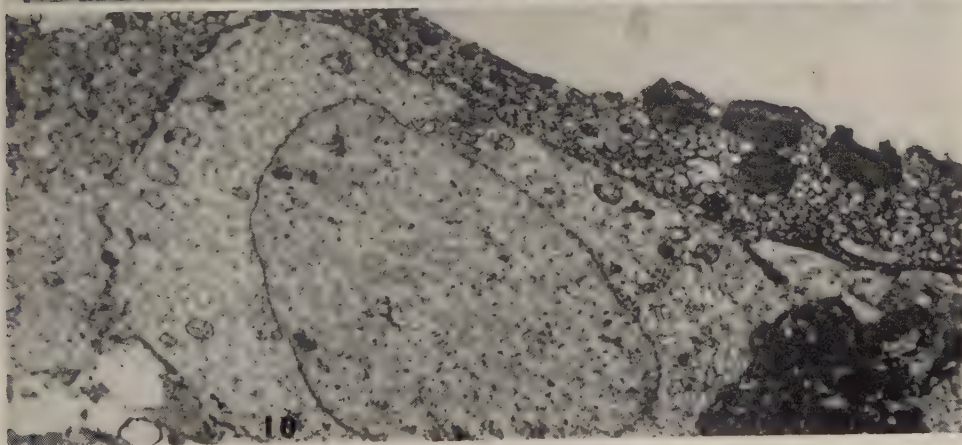
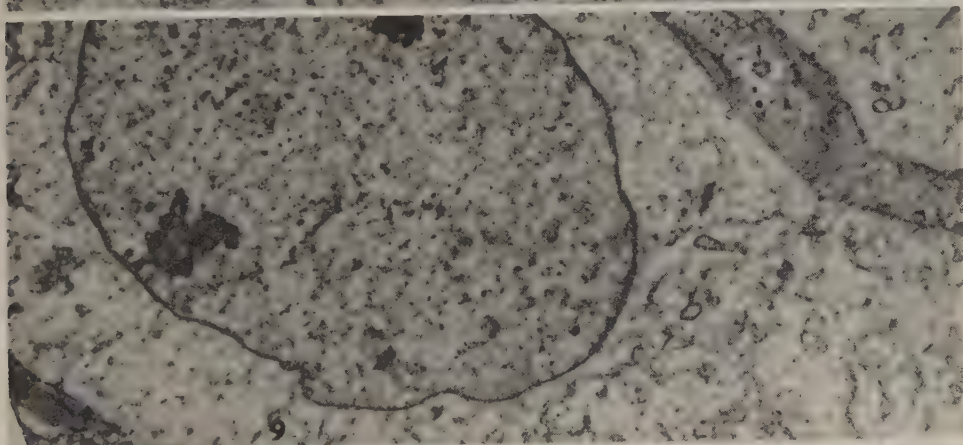
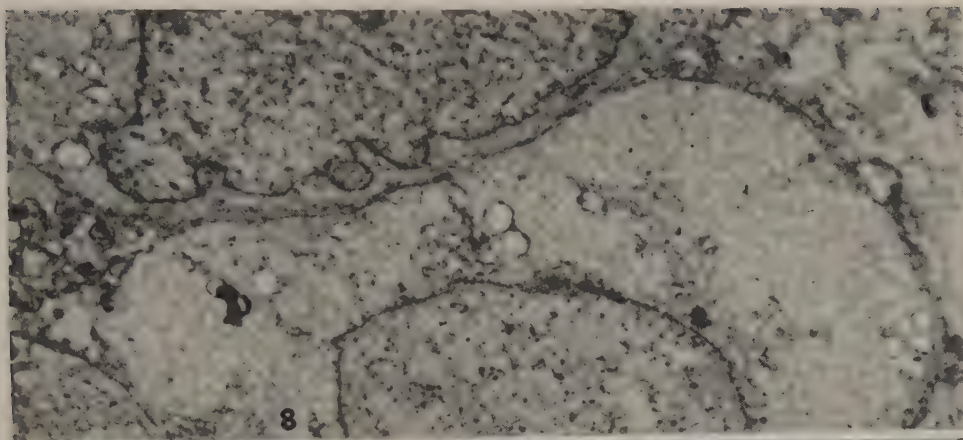


PLATE 6

EXPLANATION OF FIGURES

11 A large decidual cell from the decidua basalis. The cell possesses a fairly smooth outline, but in some places, interdigitates with the cytoplasm of contiguous cells (arrow). In the spaces between cells, there is a flocculent precipitate of ground substance and a few collagenous fibrils. Lipid droplets (L), mitochondria (M) and ergastoplasmic sacs (E) appear in the cytoplasm. $\times 10,000$

12 A cytoplasmic process of a fusiform decidual cell in the decidua vera. A large amount of matrix containing collagenous fibrils is interspersed between the cells. At the tip of the process, the plasma membrane becomes indistinct so that it is uncertain whether the fibrils are inside or outside the cell. A group of intracellular fibrils is indicated by an arrow. $\times 10,000$.

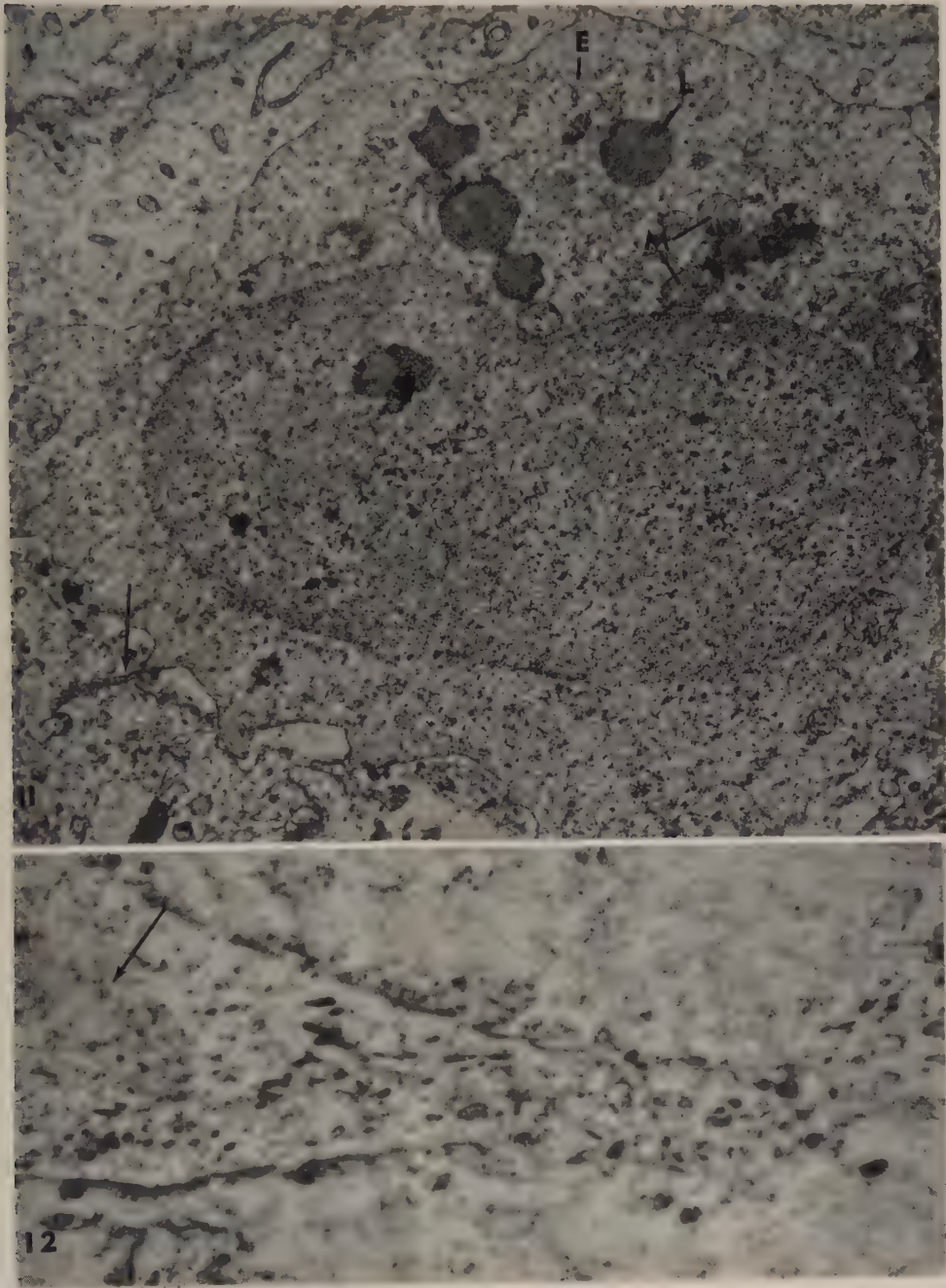


PLATE 7

EXPLANATION OF FIGURES

13 Section through the decidua basalis. A typical decidual cell is shown at the right; on the left is a portion of a polymorphonuclear leukocyte. The interstitial matrix contains precipitated ground substance and collagen fibrils. $\times 10,000$.

14 Section through a uterine gland in the decidua vera. The cell surface facing the glandular lumen (at the right) possesses numerous short microvilli. $\times 10,000$.

15 Another decidual cell which is fusiform in shape. A polymorphonuclear leukocyte is present in the lower left corner. $\times 10,000$.

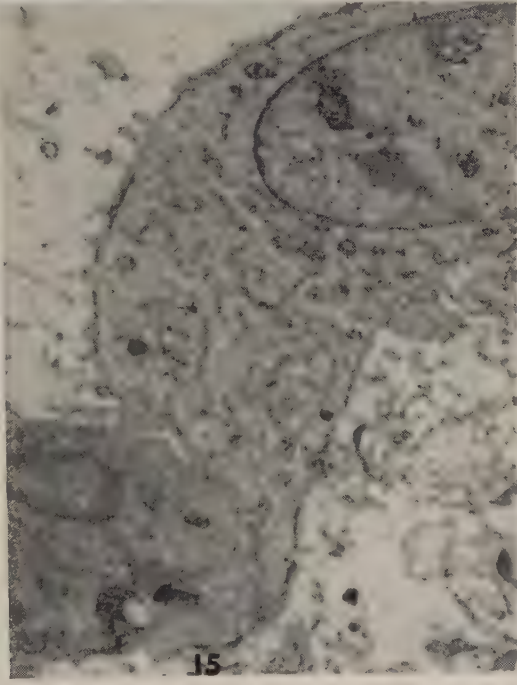
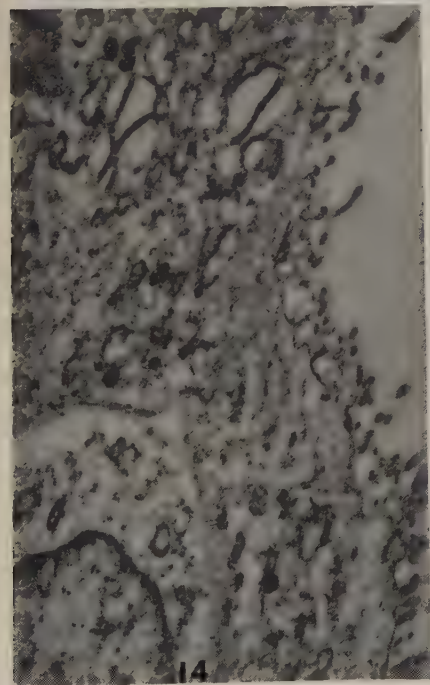
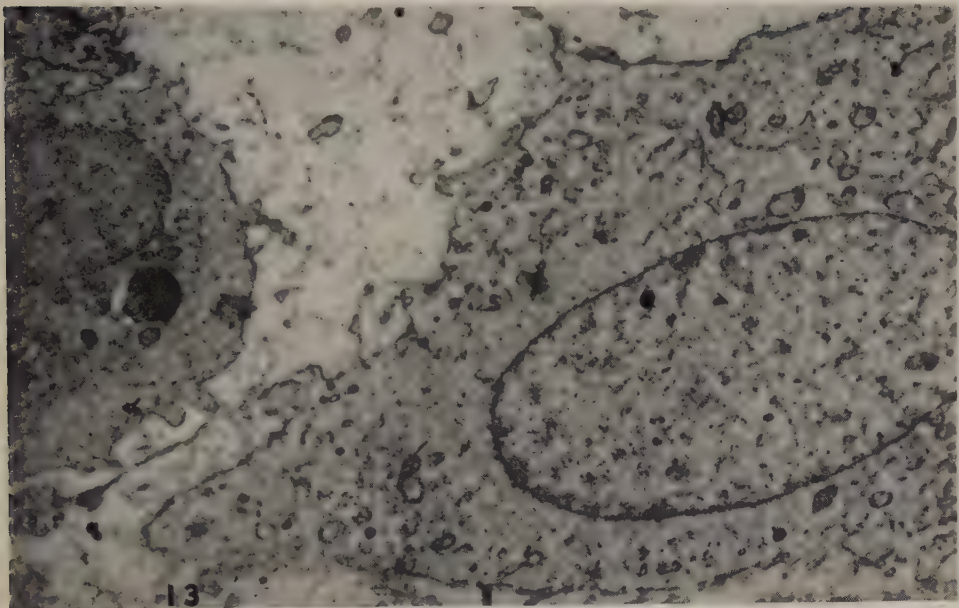


PLATE 8

EXPLANATION OF FIGURES

16 Section through a thin portion of the wall of a chorionic villus from a placenta delivered normally at term. The syncytial trophoblast has numerous slender microvilli which project into the intervillous space visible at the top of the figure. Beneath the dark syncytium is an irregular, pale zone, containing a number of mitochondria and small granules. This zone probably represents a thin cytoplasmic sheet of a residual Langhans cell (cf. figs. 19 and 20). Beneath this zone are the basement membranes of the trophoblast and the endothelium, which are separated by a connective tissue space. Wisps of collagen can be seen as fibrils in this space. Endothelium which rests on its basement membrane bounds the capillary lumen. $\times 15,000$.

17 Another view of the wall of a villus at term. $\times 7,500$.

18 Section through a similar villus illustrating a thin area over a fetal capillary. Nuclei are displaced from such regions, and become segregated in the thicker syncytium between adjacent capillaries. $\times 7,500$.

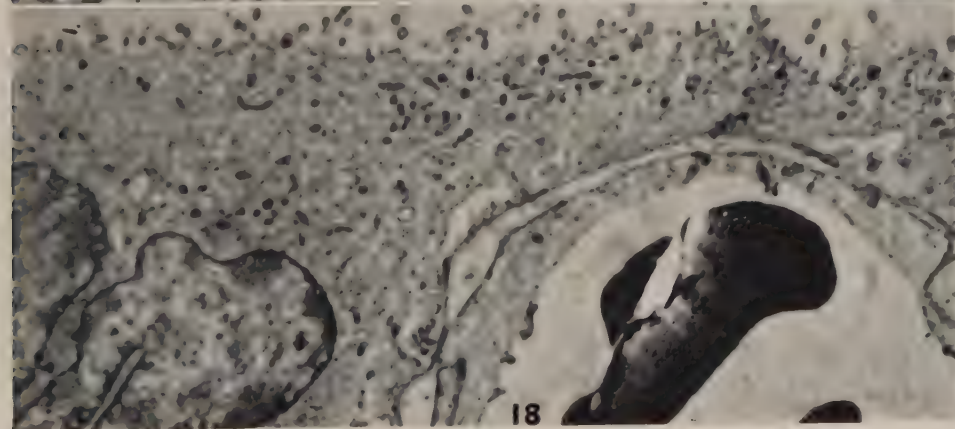
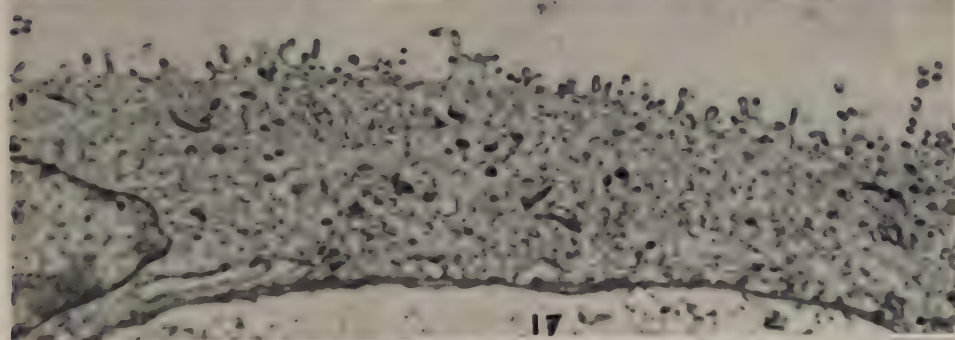
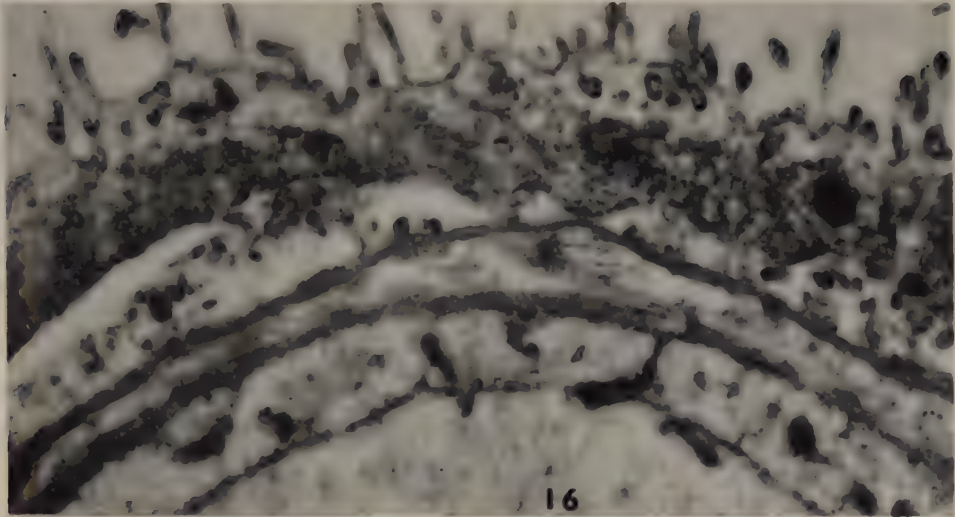
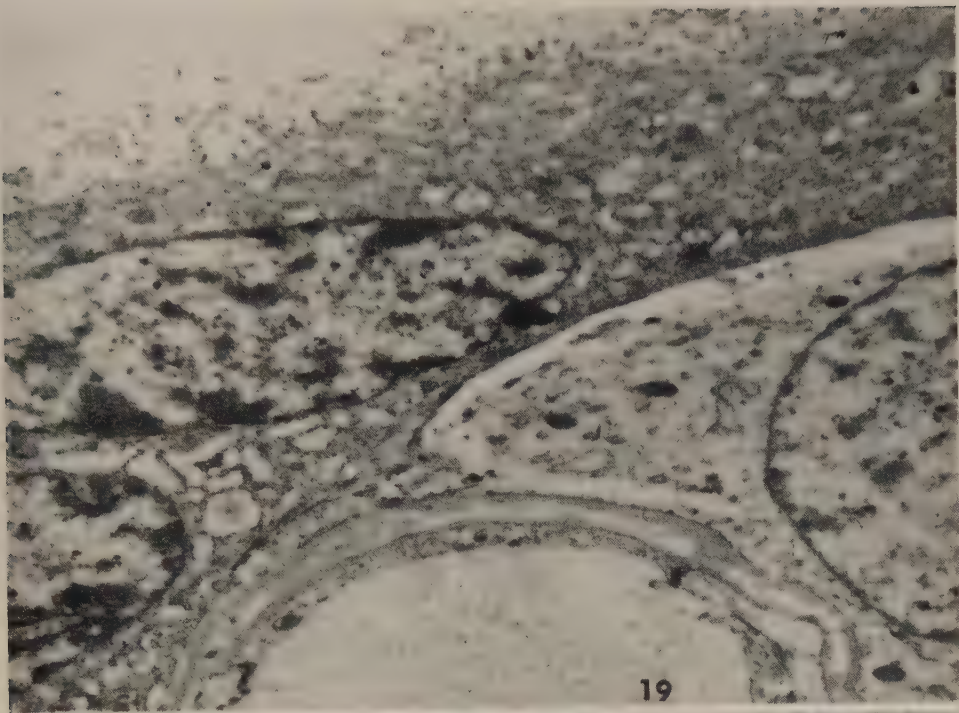


PLATE 9

EXPLANATION OF FIGURES

19 Another section through a chorionic villus. The syncytium is denser at term than at earlier stages. Fewer ergastoplasmic structures are present and those that remain appear often to be extremely dilated. A lighter cell, which is interposed between the syncytium and the trophoblastic basement membrane, is interpreted as being a Langhans cell, the flattened lateral extensions of which give rise to the appearance illustrated in figure 16. A dilated capillary is visible in the lower portion of the figure; two basement membranes with an intervening space which contains collagenous fibrils, and endothelium, separate the lumen of the capillary from the trophoblast. $\times 10,000$.

20 A similar picture showing the syncytium with a residual Langhans cell interposed between it and the basement membrane. $\times 10,000$.



REGENERATION IN THE HINDBRAIN OF NEURAL TUBE STAGES OF CHICK EMBRYOS

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SIX FIGURES

INTRODUCTION

A relatively extensive literature exists on the regeneration of the neural plate and tube of urodeles, and some studies have also been made on other species, especially chick. Harrison ('47) discussed in detail regeneration after removal of one-half of the brain anlage in neural plate stages of *Ambystoma*. He demonstrated that the regeneration takes place mainly from the contralateral side. By a rotation of the neural plate, part of the intact side was brought over to the defective side, and these cells proliferated and gave rise to the regenerating mass. Harrison also thought there was an increased mitotic activity in other parts than the operation level, even if his figures are not quite conclusive. Regeneration processes after operations at older stages of urodeles have been described among others by Burr ('16), Spirito ('30), Daltrop ('32), Detwiler ('44-'47) and Holtzer ('51). According to Daltrop the regeneration takes place mainly from the rostral and caudal ends of the operation wound, but Detwiler showed that it is from the contralateral side. Holtzer made a detailed investigation of the mode of regeneration and found that the regenerating cells almost exclusively come from the dorsal part of the contralateral side. He also studied the nuclear differentiation in detail and found that the cell material is determined to a high degree at the operation

¹ On leave from Tornblad Institute for Comparative Embryology, Lund, Sweden, on a Rockefeller Fellowship.

stages. Both Detwiler and Holtzer stressed the strong increase of the extraventricular mitoses and their significance for the regeneration process. On the contrary Harrison did not find extraventricular mitosis after extirpations at neural plate stages.

A recent review of regeneration in the brain of birds is given by Waddington ('52). Waddington and Cohen ('36) described a lack of regeneration after extirpations of neural plate material but sometimes a nearly complete regeneration after operations on 4-25 somite stages. Contrary to this, among others, Jones ('37, '42), Fugo ('40), Spratt ('40), Levi-Montalcini ('45), Rhines ('43, '44), Rhines and Windle ('44), Yntema and Hammond ('45), Levi-Montalcini and Amprino ('47), Hammond and Yntema ('47), Wenger ('50) and Birge and Hilleman ('53) obtained no regeneration after operations on neural tube stages (prosencephalon, rhombencephalon, spinal cord). Watterson and Fowler ('53) separated the right and left halves of the spinal cord of chick embryos with rapidly growing material—somites with nephrotomes—and obtained signs of regeneration in some cases. In approximately one-third of the cases a rigid mosaic development was encountered, in the rest of the cases a more or less pronounced regulation occurred. In a few cases an almost complete twinning of the spinal cord could be observed. The production of the reconstituting cells occurred nearly exclusively in the alar plate, and basal plate material was usually lacking in the regenerate. Their material did not permit an analysis of the regulation process in detail.

When operating on neuromeres in the hindbrain of chick embryos, the present author made some observations on regeneration that were later extended to an investigation of the regenerative properties of this brain part.

MATERIAL AND METHODS

The operations were made on chick embryos at stages between 7 and 18 somites. In these stages the neuromeres of the hindbrain are relatively well demarcated, and compara-

tively accurate operations can be made. Either one or two neuromeres on one side, or one neuromere on both sides, were removed from the vital-stained embryos. The operations were made *in ovo* with glass needles under aseptic conditions. The embryos were fixed in Bouin's fluid or — in a few cases — in 10% formol at different stages up to an age of $7\frac{1}{2}$ days. They were cut in 8 or 10 μ sections — usually transversely — and were stained with Delafield's hematoxylin and eosin. In some cases, wax-plate reconstructions were made. Altogether 49 embryos were fixed after unilateral extirpations. Twenty-one of them were fixed during the first 12 hours after the operation, 28 of them at an age of $4\frac{1}{2}$ to $7\frac{1}{2}$ days. Eight cases of bilateral operations were fixed.

RESULTS

I. Wound healing during the first 12 hours after a unilateral operation

In all cases the first rhombomere was extirpated. Three embryos were fixed during the first two hours after the operation. No signs of regeneration at all could be seen in them. Six embryos were fixed between two and 6 hours after the operation. In all cases ventral and sometimes dorsal outgrowths from the contralateral side could be seen. In plate 1, figure 2, a section through the operation level of such an embryo is seen. Twelve embryos were fixed between 6 and 12 hours after the operation. Two of them showed no signs of regeneration, in three cases (6 to $6\frac{1}{2}$ hours after the operations) there were small gaps left on the operated side of the neural tube, and in 7 cases the operated side had regenerated more or less completely. In plate 1, figure 3, a section through such an embryo is shown.

It was thought to be of interest to study possible changes in the mitotic activity of the contralateral side of the brain. The following method was used:

The number of mitoses and the volume of the brain wall (= cell layer) was calculated from 10 successive sections of

the intact side, level with the operation (first rhombomere). The rate $\frac{\text{mitotic number}}{\text{volume of the wall}}$ was calculated, here called "relative mitotic rate," and the rate was compared between operated and normal embryos. The method is justified by the following

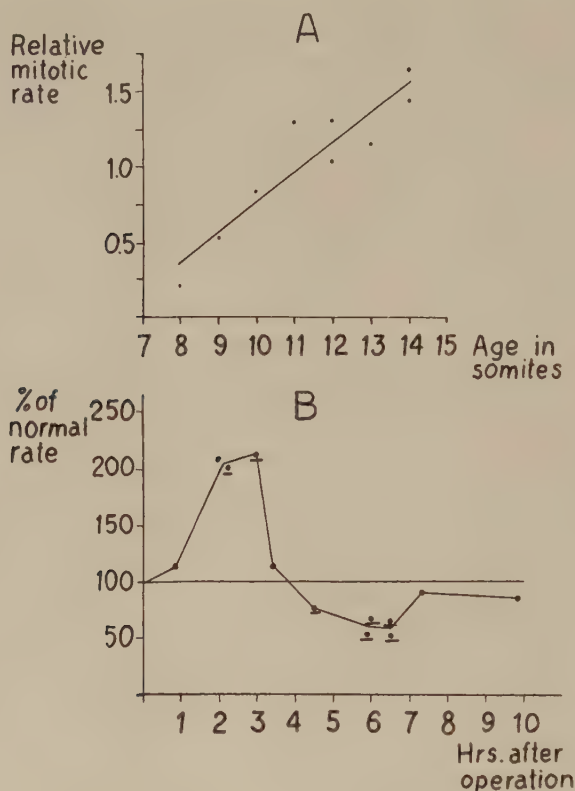


Fig. 1 Graphs illustrating the changes in relative mitotic rate. A: The rate of normal embryos plotted against their age in somites. B: The rate in operated embryos, expressed as percentage of the normal mean rate for each stage, and plotted against the time elapsed after the operation. The underlined points mark values that are significantly different from the normal population ($P < 0.01$).

fact: the rate is compared only between embryos belonging to the same age group, where the number of cells contained in a certain volume of the brain wall can be supposed to vary only randomly in different individuals. It can further be

supposed that the percentage of cells, too far differentiated to divide, is the same. The volume of the brain part studied thus gives a good idea of the actual number of cells capable of mitosis.

The value of the "relative mitotic rate" for normal embryos, varying in age between 8 and 14 somites was calculated from 9 embryos. The result is shown in text-figure 1 A, where the rate is plotted against the somitic age. As is apparent from this figure, the rate increases as an approximately straight line with the increase in somite number. A regression line of the first degree was therefore fitted to the values with the following result: rel. mitotic rate $= 0.197$ (somitic number) $- 1.21$.

The rate for 12 operated embryos was then calculated and expressed as a percentage of the rate in the normal embryos of the same age, obtained from the formula above. In text-figure 1 B the results are plotted against the time elapsed after the operation. The normal rate thus represents the horizontal line 100%. In the figure the graph corresponding to the operated embryos seems to deviate from this line. The underlined points in the figure mark values significantly different from the normal population ($P < 0.01$).

A peak of mitotic activity is thus present two to three hours after the operation. The rate then decreases rapidly to the normal level and goes even below that level about 6 hours after the operation. The peak in the operated embryos thus compares well with the outgrowth of the regenerated mass.

A proliferation of course takes place within the regenerating mass, too. No increase in extraventricular mitosis could be observed, contrary to Detwiler's and Holtzer's results on urodeles.

II. Fate of the regenerated part

For this study the 28 embryos, fixed at an age of $4\frac{1}{2}$ to $7\frac{1}{2}$ days, were used. At this stage the formation of the brain

nuclei is well under way — the migration areas and layers are formed and the nuclei have begun to form from them.

Nearly half of these embryos showed a relatively normal morphology of the regenerated part, as far as could be seen from the hematoxylin-eosin stained sections. In plate 1, figure 4, a section through such an embryo is shown. In this case the left first rhombomere was excised at the 10 somite stage, and the embryo was fixed at an age of 5 days 17 hours. On the intact side the 4 longitudinal columns, described in detail by Hugosson ('55) can be seen. In the ventro-lateral column a second migration layer has been formed. Dorso-medially to it a medial longitudinal fascicle is situated. On the operated side, the same structures can be found, but the cell layers are distinctly hypoplastic. Also in the 11 other cases belonging to this group a normal or very nearly normal morphology of the cell areas could be seen, but a more or less pronounced hypoplasia was present. This hypoplasia might have been regulated during the further development.

In the rest of the embryos (16 out of 28), more or less well-developed regenerates could be found. The morphology and histology was often strongly abnormal. The cell layer might be only a thin ependymal stratum, a matrix layer without any peripheral cells or a matrix layer with fairly normally migrated cells. In some cases, where the cellular areas were fairly normal, the wall showed an abnormal folding. In other cases the dorsal and ventral halves of the wall were not or only incompletely fused. In a few cases the external limiting membrane was broken through by brain fascicles, resembling aberrant nerves.

In some cases, the morphology of the brain parts surrounding the operation level was abnormal. The abnormality consists of an abnormally strong folding of the wall, which was always unusually rich in mitoses. In two cases the mesencephalon was strongly folded, and in one case the metencephalon (the second neuromere had been excised).

III. Results of bilateral extirpations

Bilateral extirpations were made in order to see if regeneration could take place from the rostral and caudal margins of the wound.

In one case the rostral and caudal ends healed up separately, leaving a gap between them, and in two cases only a very narrow connection existed. In the rest of the embryos the two parts fused more or less completely, but the extirpated part was absent. No signs of regeneration could thus be seen, which was in agreement with the findings of Levi-Montalcini and Amprino ('47). In plate 1, figure 5 a reconstruction of such a brain is shown, demonstrating the shortening of the medulla oblongata, due to the absence of the second rhombomere.

A very typical abnormality of the rhombencephalon could be seen on the operation level in most cases, consisting of the absence of the median longitudinal fissure and the septum medullae. It resembles very much the picture obtained by many authors (i.a., Lehmann, '28) in amphibians, when the notochord was extirpated in early stages. Waddington ('52) mentioned that similar observations have been made on avian embryos. In the present cases it was probably caused by dislocation of, or damage to, the notochord at the operation.

The rostral part of the brain was heavily deranged in three cases, and slightly so in one case. The abnormality consists of a folding, corresponding to that described above for some unilateral operations. In the three cases where the first rhombomere was extirpated bilaterally, the folding of the archencephalon was strong (plate 1, figure 6). The forebrain and the midbrain were most affected and were distinctly smaller than normally. Caudal to the operation level the anatomy was normal.

DISCUSSION

It can be stated that regeneration can take place after unilateral operations, consisting of extirpation of one rhombomere in chick embryos in 7 to 18 somite stages. This finding

is thus in accordance with Waddington and Cohen ('36) and Watterson and Fowler ('53) but not in agreement with the results of Spratt ('40), Yntema and Hammond ('45), Hammond and Yntema ('47) and Birge and Hilleman ('53). The fact that only a small lesion was made by the present author might explain the high frequency of regenerating cases. Birge and Hilleman made lesions exactly corresponding to those made by the author, but they used electrocoagulation instead of extirpation with glass needles, which probably explains the difference in results. Harkmark's ('54) operations were also made with electrocoagulation on older stages (4 days and older), and he obtained no regeneration.

The regeneration process can be described in the following way: On the intact side an increase in mitotic activity occurs. Cells formed by the proliferation migrate into the brain defect, filling up the gap. Possibly a mechanical upfolding helps to bring the wound edges together. The cell migration seems to take place both ventrally and dorsally; the ventral migration in most series seems to be the strongest one. In some cases no wound healing at all took place.

In nearly half of the cases surviving at an age of $4\frac{1}{2}$ to $7\frac{1}{2}$ days, the regenerated mass was organized in a fairly normal way, as far as could be found in hematoxylin-eosin stained material. It is very likely that in older stages and with the aid of special staining methods it would be possible to demonstrate defects in the case of certain special neurons, as was the case in urodeles according to, among others, Detwiler ('44) and Piatt ('49). In the stages fixed, the operated side showed a slight but distinct hypoplasia. It might be regulated during the further development.

According to these observations the rhombencephalon of chick embryos at the stages in question does not represent a rigid mosaic, but can regulate at least small defects.

In two-thirds of the cases, the regenerate was abnormal morphologically or histologically. The abnormalities found suggest that the forces governing proliferation and differentiation of the cells have been disturbed at the operation.

Of special interest is the abnormality observed in the brain parts at some distance from the wound, especially in parts lying rostral to it. They seem to be most strongly developed after bilateral operations but are quite distinct in a few unilateral operations also. They consist of a reduction of size, an abnormally strong folding, and an accumulation of mitoses. Harrison ('47) suggested an increase in mitotic activity in parts other than the regenerating one. The abnormality described here is undoubtedly correlated with an increase in mitotic activity. As it is most conspicuous after bilateral operations—when no regeneration takes place—it cannot be the result of the regeneration itself. Similar abnormalities can be found spontaneously. The present author found one chick embryo with a folding of the mesencephalon, quite comparable to that obtained after unilateral operations. Patten ('52) described phenomena in human embryos, similar to the folding demonstrated in plate 1, figure 6. He called it "overgrowth." In the cases, discussed in the present paper, the high mitotic number suggests an overgrowth, but, on the other hand, the size of the brain part is strongly reduced. A likely explanation seems to be that fewer cells than normally differentiate, which would give an increase in mitotic number and a folding as a result. The growth of the brain volume, however, is greatly dependent on the cell differentiation process, whereby a reduction of the final size would be explained.

SUMMARY

1. The hindbrain of chick embryos between 7 and 18 somite stages is capable of regeneration when one-half of one or two rhombomeres is extirpated.
2. The regenerating cell mass develops in a normal way in nearly half of the cases, surviving at an age of $4\frac{1}{2}$ to $7\frac{1}{2}$ days. In the rest of the cases abnormalities of different kinds develop.
3. No regeneration occurs after bilateral operations.

4. The regeneration is correlated with an increase in mitotic activity on the intact side. Cells migrate into the gap via the floor and partly via the roof.

5. In some cases abnormalities, consisting of strong folding, occur in other parts of the brain than the regenerating ones. They seem to be an unspecific operation effect.

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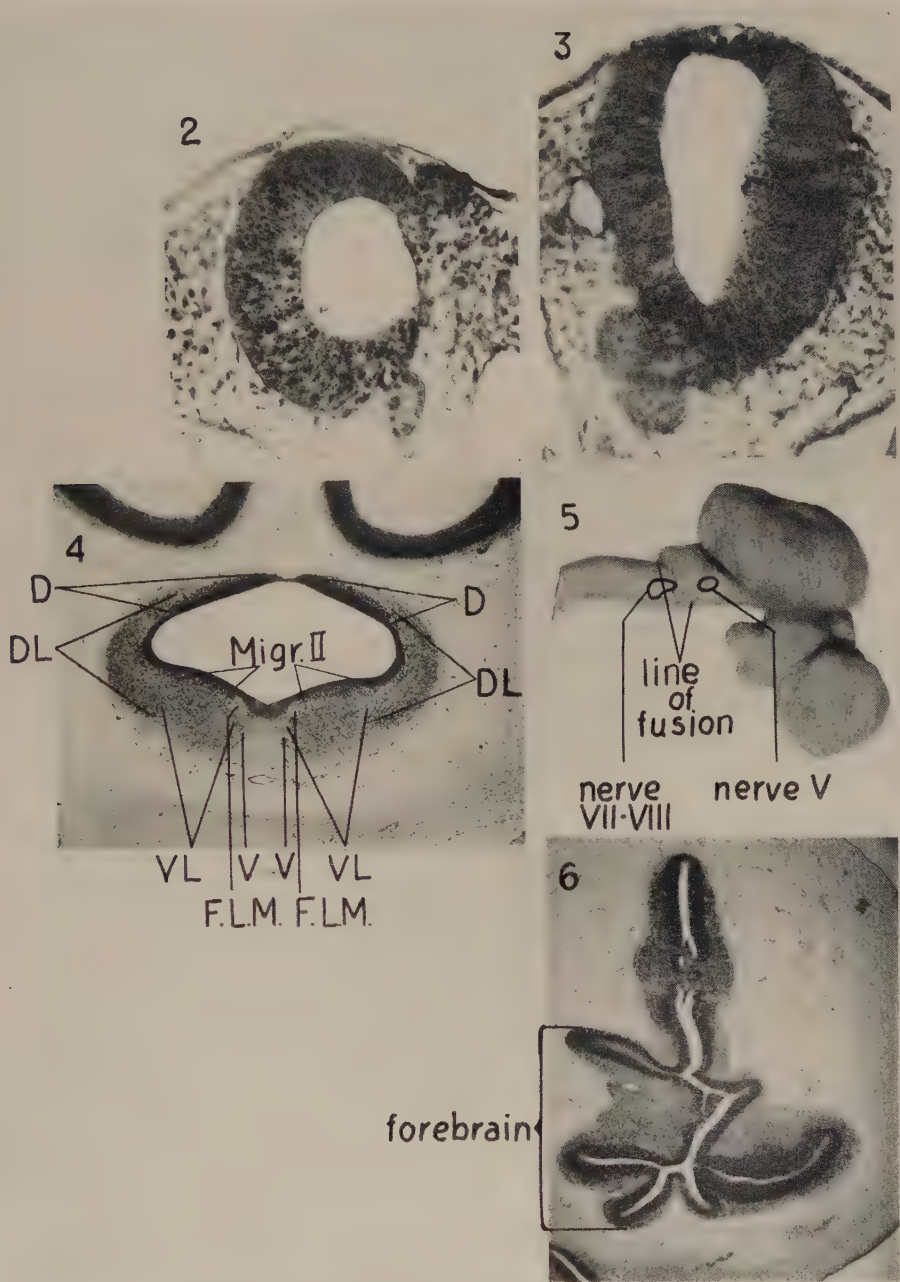
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PLATE 1

EXPLANATION OF FIGURES

- 2 Transverse section through the operation level in an embryo, where the right half of the first rhombomere was extirpated at the 12-somite stage. The embryo was fixed 5 hours after the operation. Dorsal and ventral outgrowths are distinct. $\times 160$.
- 3 Transverse section through the operation level in an embryo, where the left half of the first rhombomere was extirpated at the 15-somite stage. The embryo was fixed $8\frac{1}{4}$ hours after the operation. The brain wall has regenerated. The notochord was split at the operation and is therefore duplicated. $\times 160$.
- 4 Transverse section through the operation level in an embryo, where the left half of the first rhombomere was extirpated at the 10-somite stage. The embryo was fixed at the age of 5 days 17 hours. The cell areas of the operated and the intact sides are quite comparable. The operated side is hypoplastic. D = dorsal cell column, DL = dorso-lateral cell column, VL = ventro-lateral cell column, V = ventral cell column. Migr. II = second migration layer in VL. F.L.M. = fasciculus longitudinalis medialis. $\times 25$.
- 5 Wax-plate reconstruction of an embryo, where the second rhombomere was extirpated bilaterally at the 12-somite stage. The embryo was fixed at an age of 6 days two hours. The shortening of the medulla, due to the absence of the second rhombomere, is visible. $\times 7$.
- 6 Horizontal section through the forebrain of an embryo, where the first rhombomere was extirpated bilaterally at the 11-somite stage. The embryo was fixed at an age of 5 days 12 hours. The strong folding of the forebrain is distinct. $\times 16$.



A METHOD FOR FETAL HYPOPHYSECTOMY BY DECAPITATION IN THE RAT

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TEN FIGURES

Despite the vast accumulation of information during the past few decades concerning the interrelations of the endocrines of vertebrates in the postnatal period, relatively little is known regarding this problem during prenatal development. Considerable evidence has accumulated in the last few years to show that some of the embryonic or fetal endocrine organs of vertebrates produce substances which react in a specific manner not unlike the hormones secreted by these organs during the postnatal period. Nevertheless, it is not yet known whether the function of these prenatal substances is similar to or the same as that of the now well known postnatal hormones. Does the embryonic or the fetal pituitary play the role in prenatal life it is destined to perform postnatally? In other words, are the adrenals, the gonads, or the growth of the fetus under the control of trophic substances or hormones elaborated by the pituitary or do they develop independently of hormones or hormone-like substances?

One of the methods available to us in attempting to determine the function of fetal endocrine organs is that of removal or destruction. The pioneer work was done by Allen ('16) and Smith ('16) who working independently, utilized the surgical method on the frog. The following year Allen ('17)

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published results of similar experiments on newts followed by Humphrey ('33) on *Amblystoma*. In 1919 B. Wolff published an account of the decapitation of a rabbit fetus *in utero*. This headless fetus was dead 15 hours after the operation.³ Some years later Et. Wolff and Stoll ('37) in attempting to produce experimental cyclocephalids destroyed the primordium of the encephalon of chick embryos of 40 hours incubation by x-ray irradiation. Fugo ('40) reported experiments in which hypophysectomy was performed on chick embryos by surgery. In 1943 Raynaud succeeded in provoking lesions in the pituitary of mouse fetuses by x-rays and a few years later Wells ('47) surgically decapitated rat fetuses, Jost ('47), rabbit fetuses, Foote and Foote ('49), hamster fetuses, and Case ('51), chick embryos.

In performing fetal hypophysectomy some investigators have removed the fetus from the uterus, severed the head, being careful not to disturb the placenta, and left the decapitated fetus in the abdominal cavity of the mother. Another method developed and described in this paper consists of leaving the decapitated fetus in the uterus. This procedure while more difficult than the preceding allows development of the decapitated fetus in its natural environment since only the part to be removed is exteriorized.

The rabbit and the rat seem to have been the forms most frequently employed in attempts at fetal hypophysectomy by decapitation. The rabbit offers greater facilities for such an operation than does the rat by virtue of its size and the greater thickness and consistency of the wall of the uterus. However, these advantages are partially offset by the vastly greater precautions necessary to avoid infections in the rabbit following abdominal surgery as contrasted with the rat. In a few instances hamsters have also been successfully decapitated during fetal development. No one has reported success in decapitation experiments on fetal guinea pigs and in the few attempts we made to decapitate fetal guinea pigs

³ Two German investigators, Zander (1890) and Alexander (1892) reported that the destruction of the whole encephalon during the development of the adrenals had a stunting effect on these structures.

they were found to be more refractory than rabbits or rats in that abortion usually followed the operation.

In a recent paper Wells ('50) describes a method for delivering of the rat fetus from the uterus and introducing it into the maternal peritoneal cavity. Through an incision in the uterine wall, the fetus is gently expelled without exteriorization of the placenta. This method is delicate and as pointed out by the author the ensuing mortality is very high. Furthermore, the operation cannot be performed until near the end of gestation.

Our first attempts at decapitation were made on the rabbit. The method we employed consisted essentially of keeping all but the head of the fetus in the uterus during and following the operation. By modifying the method used by Jost ('47) in his castration experiments on fetal rabbits we were able to apply it with success in the decapitation of the rat fetus. Because of its importance in the possible solution of a variety of problems it was considered desirable to describe our procedure. The method can be used with little or no modification in other mammals and should be of value particularly to those interested in fetal endocrinology.

THE METHOD

A. Anesthesia

Ether anesthesia was generally used although sodium nembutal (30 mg in 1 cm³ sterile water per kilogram body weight) was also found to be satisfactory. However, individual reactions to this barbiturate may sometimes yield undesirable consequences. We never used an anesthetic for the fetus itself, a practice recommended by some investigators but found unnecessary in our procedure.

It was not necessary to administer an antispasmodic to pregnant rats since abortion very rarely occurred in our experiments. The surgical manipulations of the uterus necessary for decapitation apparently do not cause contractions sufficiently violent to provoke abortion. Dead fetuses are

either retained in the uterus and resorbed or are expelled at the time of parturition. Decapitated fetuses were usually recovered at term by Cesarean in order to avoid loss through cannibalism.

B. Technical procedure

1. *Exposure of uterine horn.* Following the anesthesia the pregnant female is tied back down on an operating board. The abdomen is shaved or clipped and the denuded area sterilized with tincture of zephiran. A medial abdominal incision, approximately 4 cm in length, is made through skin and muscles and held open by means of hemostatic forceps. The desired portion of the uterine horn, preferably near the cephalic end, is then gently pulled through the incision and disposed upon tepid saline-moistened gauze which is adjusted so as to prevent the return of the exposed segment into the abdominal cavity (fig. 1).

2. *Location and exteriorization of head.* The antimesometrial wall is very thin and translucent so that it is relatively easy to detect the outline of the fetus within the uterus and thus to locate its head. The head is then gently grasped and held between thumb and forefinger of the operator's left hand (fig. 2). With a small needle-holding forceps a chromic catgut (no. 5-0) is inserted into the antimesometrial wall in the area surrounding the head (fig. 2 a). The size of the circumference, which will depend upon the age of the fetus, should be ample but not excessive and for an 18-day fetus its diameter should be approximately 8 mm. In our early experiments 6 stitches were usually taken in the area thus delimited, as shown in figures 2 and 3. However, in subsequent experiments we found it advisable to make no more than 4 stitches in order to prevent undue tearing of the uterine wall. The two ends of catgut *a* are then joined in a single loose tie and an incision made through the uterine wall within the circle (fig. 3). The incision should not only include the uterine wall but must also cut through the chorioallantoic membrane. The latter should be cut with fine scissors care-

fully avoiding, however, severance of blood vessels. When the top of the head is apparent gentle pressure is applied with the thumb and forefinger of the operator's left hand while the assistant holds the two extremities of catgut *a* ready to tighten them as soon as the head has been expressed (fig. 4). The shoulders and forelimbs of the fetus must be kept within the uterus and not be allowed to slip out of the incision when the head is being expressed. This can easily be avoided by tightening the tie in catgut *a* at the proper time. This is very important since it is extremely difficult to put exteriorized parts back into the uterus and it is virtually impossible to return the totally exteriorized fetus without serious injury. Every precaution should therefore be taken to avoid this contingency by tightening as quickly as possible suture *a* after the exteriorization of the head.

3. *Decapitation.* A loop of heavier catgut, no. 3-0 or 4-0 depending upon the age of the fetus, is passed around the neck of the fetus and carefully tightened (figs. 4 and 5 b). The younger the fetus the more cautious must one be to avoid severing the head when tightening this ligature. It is also important when placing the ligature around the neck to avoid membranes or part of the antimesometrial wall. When the loop has been properly placed and tightened the head is severed with small, curved scissors, approximately 2 mm above the ligature, which is allowed to remain in place (fig. 5).

4. *Suturing the uterus.* Following removal of the head a fine catgut is inserted into each lip of the uterine incision at two diametrically opposite points about equi-distant between the two ends of the incision (fig. 6 c). It is important when inserting catgut *c* not to stitch any part of the embryonic membranes. The two ends of this stitch should be long enough to allow the operator to hold them by means of forceps 3 to 4 cm above the incision and by gentle upward pulls, the neck of the fetus usually slips back into the uterus (figs. 7 and 8). If it does not slip back freely slight pressure on the cut surface of the neck with a narrow spatula or other blunt in-

strument will usually accomplish this objective.⁴ Then while continuing to hold the ends of ligature *c* with forceps the first loop *a*, which was inserted in the antimesometrial wall, is loosened and removed (fig. 7 a). While suture *c* is being held taut the two lips of the uterine incision are closed with interrupted sutures of fine (no. 5-0) catgut (figs. 8 and 9). Fragments of the lips of the incision through the fetal membranes may bulge out between the stitches. When this happens the exteriorized fragments are pushed back with a fine pair of forceps or cut off and the aperture closed with an additional suture.

5. *Placing the uterus back in the abdominal cavity.* The moistened gauze upon which the exposed horn of the uterus was disposed is removed and the uterine segment pushed back into the abdominal cavity (fig. 10). After the wound has been cleaned muscles and skin are sutured in the customary manner.

We have employed the procedure outlined above in subtotal decapitations as well as in cases where we attempted to destroy the pituitary by cauterization. However, in these operations we remove the neck ligature following the operation. The latter operations are important where one is interested in preserving parts of the skull or the jaws. The likelihood of involving the thyroids is also eliminated in subtotal decapitations.

Following these operations rats were placed into small individual cages and usually given access to food and water immediately. They were kept in a warm room for the next 24 to 48 hours. Unlike rabbits they did not require the administration of glucose postoperatively nor was it necessary to administer an antispasmodic.

⁴ When the embryo does not slip back freely, or under slight pressure, one usually finds that the embryonic membranes have been sewed to the lips of the uterine incision by catgut *c*. In this case a second catgut *c* should be inserted to either side of the first, being careful to avoid embryonic membranes, and the first removed.

GENERAL RESULTS

Fetal hypophysectomy by decapitation does not appear to affect appreciably the growth and development of the rat fetus. The decapitated fetus at term has the size and general appearance of its litter mate control and its body movements at this time are similar to those seen in normals. We observed no malformations of the body or of the extremities except for an occasional pronounced edema of the entire body. Wells in discussing the paper of Jost ('53 p. 414) states that "the weight of decapitated fetuses seems to be significantly less than that of controls decapitated at autopsy." In our experiments the size of the body and the appendages of decapitated fetuses was in general not conspicuously different from that of controls at the time of parturition.

Perhaps the most evident effect of decapitation was noted in the adrenals. These were smaller in decapitated fetuses, a difference which could usually be detected at autopsy, particularly in those fetuses which had experienced the longest period of hypophyseoprivia. This observation was confirmed by microscopic examination. Experimental adrenals showed a decrease in the width of the cortical zone. These observations confirm the work of Wells ('50) and Kitchell and Wells ('52) who as a result of their observations, emphasize the role of the pituitary in the normal activity of the fetal adrenal.

With respect to the development of the gonads and sexual differentiation we did not find conclusive evidence of a hormonal deficiency in any of our decapitated rat fetuses. The genital tract of fetuses decapitated on the 16th day and later appeared to be normal when examined on the 21st day. This observation is in accord with the results of Jost and Colonge ('49) and of Wells ('50) in the rat. The reduction of the testicular interstitial cells observed by Wells did not appear to be very evident in our experiments and is being further investigated.

DISCUSSION

In order to determine the effect of hypophysectomy following decapitation it is obviously important to perform the

operation as early as possible. The method here described does not permit a successful decapitation of the rat fetus prior to the 15th day of gestation owing to the difficulty of securing a tight ligature around the neck before tissues have acquired sufficient consistency. When a ligature cannot be applied the neck may be compressed for an instant with a blunt forceps and then severed cranially. Decapitation may be readily performed by the method described on the 16th and 17th days of gestation and it is relatively easily performed on or after the 18th day.

In an excellent review of some problems of fetal endocrinology Jost ('53) pointed out that the question of the dependence of the rat fetal testis upon gonadotrophic stimulation is not completely resolved and requires further research. The same author in his brilliant studies on the fetal hypophysis of the rabbit has shown that the hypophysis appears to be necessary for normal sexual differentiation since modifications appear in the genital tract when decapitation occurs prior to the 24th day of fetal life. He was able to demonstrate in the rabbit by the application of the McManus-Hotchkiss histochemical method a rather sudden hypophyseal discharge between the 22nd and 24th days of gestation indicating the onset of hypophyseal secretory activity early in the last quarter of fetal life. The reader is referred to a recent review by Jost ('54) for a full account of the effects of fetal decapitation and their interpretation.

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PLATE 1

EXPLANATION OF FIGURE

- 1 Drawing showing exposure of uterine horns for selection of fetus by median abdominal incision.

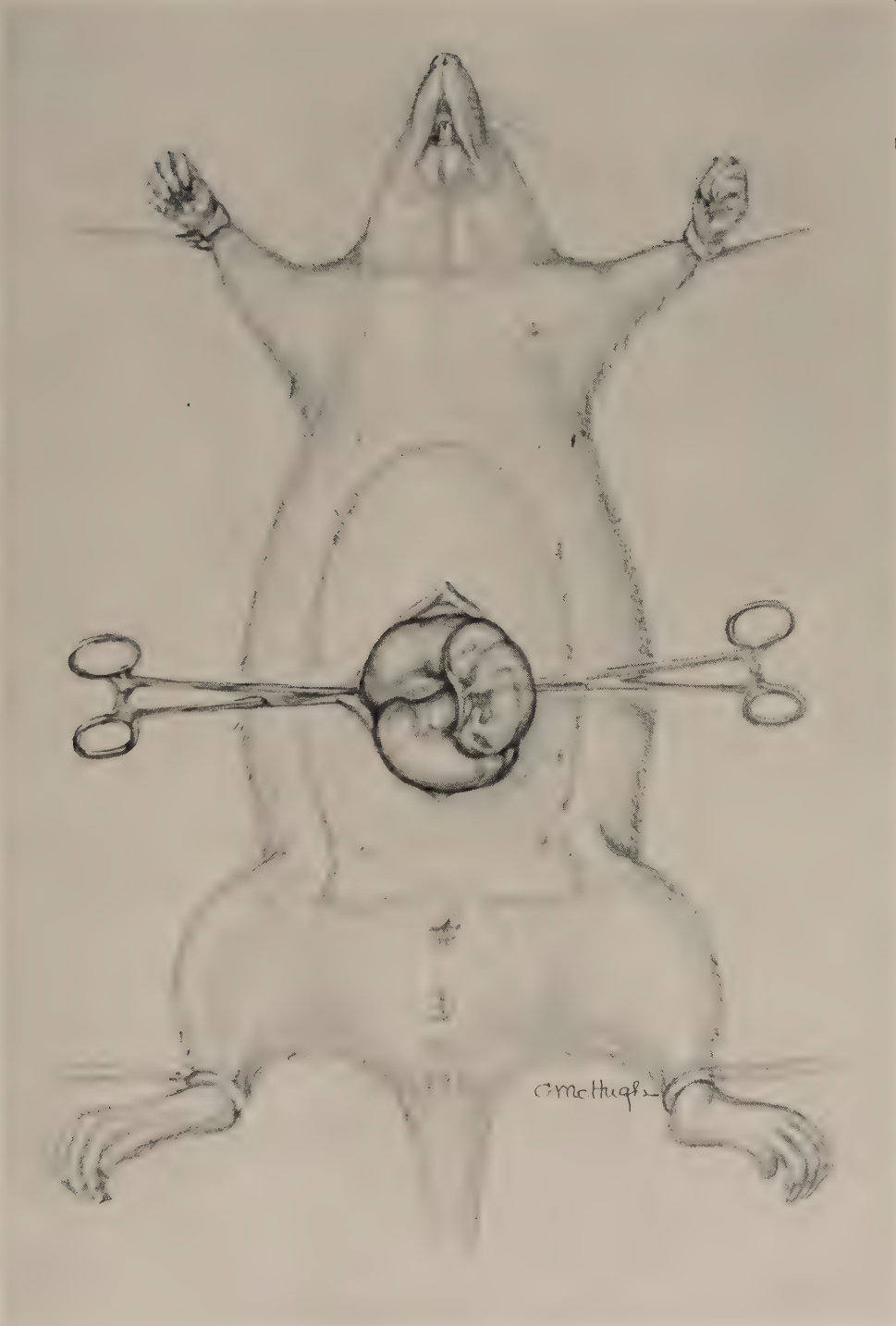


PLATE 2

EXPLANATION OF FIGURES

- 2 Drawing showing position of purse string suture (c) placed in wall of uterine sac and site of incision.
- 3 Shows incision for exteriorization of head. The two ends of catgut (a) are joined in a single loose tie.
- 4 Shows exteriorized head and catgut (a) held taut to avoid exteriorization of rest of fetus. A loop of catgut (b) is shown in place around the neck slightly below base of the skull.
- 5 Head is being severed while catgut (a) continues to be held taut and the loop formed by (b) has been tightened to avoid excessive hemorrhage.

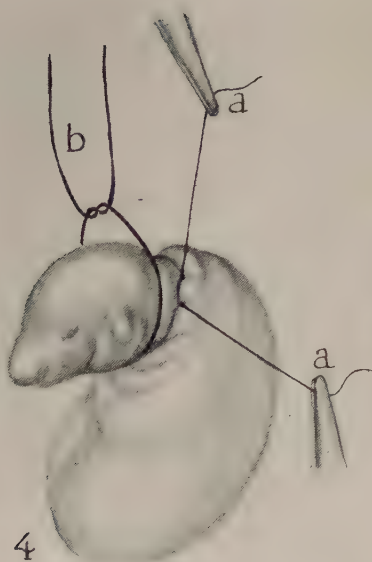
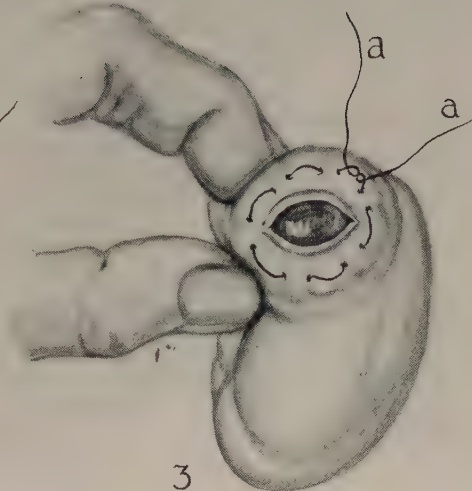


PLATE 3

EXPLANATION OF FIGURES

- 6 The loop around the neck, the ends of which are shown, (b) is allowed to remain in place. A fine catgut (c) is shown being inserted in each lip of uterine incision.
- 7 The ends of catgut (c) are being held while the first purse string suture (a) is loosened in preparation for its removal.
- 8 Shows catgut suture (c) and manner in which it serves to depress neck when held taut.
- 9 Neck is completely depressed in uterine sac. Suture (c) has been tied and ends are being held taut while incision is closed with interrupted sutures.

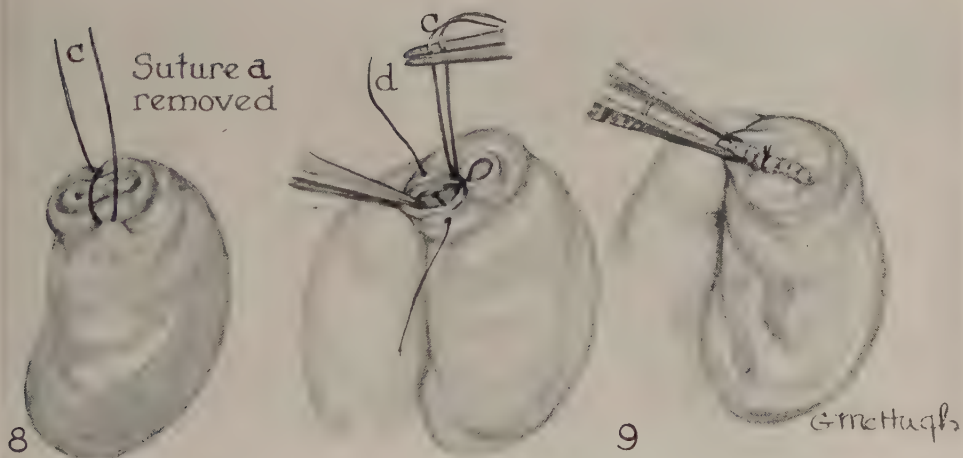
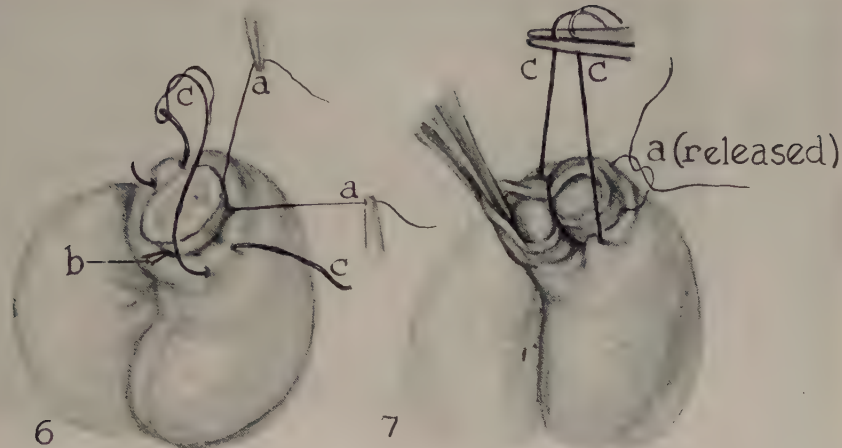
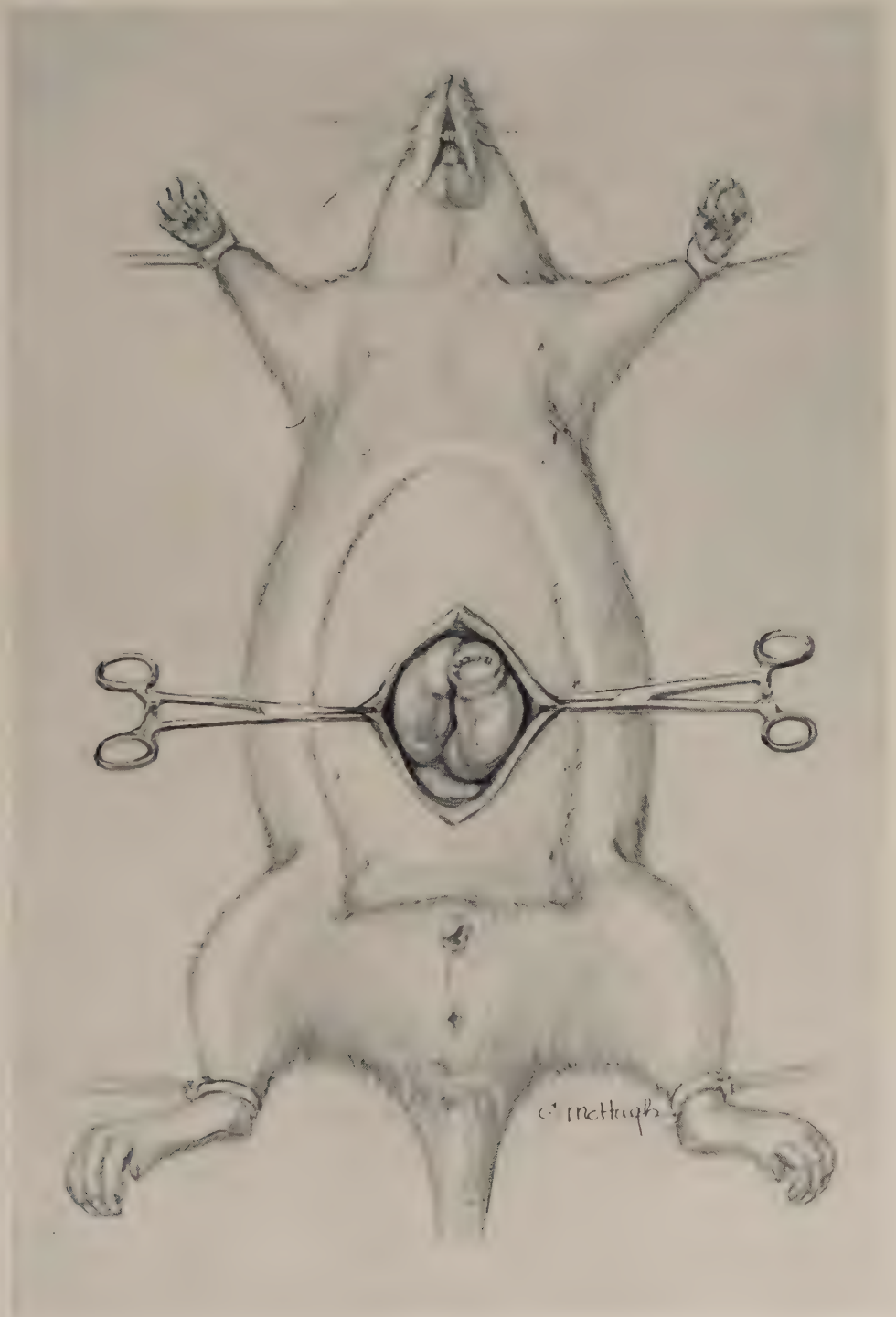


PLATE 4

EXPLANATION OF FIGURE

- 10 Uterine sac containing decapitated fetus shown in abdominal cavity prior to suturing of abdominal incision.



EFFECT OF VAGUS NERVES ON HEART RATE OF YOUNG DOGS: AN ANATOMIC-PHYSIOLOGIC STUDY ¹

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FOURTEEN FIGURES

The influence of the vagus nerves upon the heart and heart rate of the adult mammal has attracted innumerable investigators. Their combined efforts have provided us with precise and generally accepted information on the respective physiologic role of both the left and right vagus nerves in the mature animal (see Hamaty and Truex, '54). There also has been general agreement, though to a lesser degree, regarding the influence of the vagus nerves on the heart of the newborn animal.

Wiggers ('23) has stated, "To judge from the rapid rate in infancy, its gradual decrease during childhood, and the fact that atropine does not accelerate it in children, the vagus influence has not developed in early life but is acquired gradually." At a later date he wrote, "The heart slows gradually from infancy to adult life due to increasing vagal dominance over the sinoatrial node" (Wiggers, '49). Windle ('40) noted that most investigators who have studied the phenomenon of vagus inhibition in young postnatal mammals have been able to produce slowing or cessation of the heart beat by stimulating the peripheral cut end of the nerve, but in many instances the effects seem to have been weaker than would have been

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obtained in adults. He also pointed out that the nearer to birth, postnatally, the less chance there is of producing inhibition by stimulating the vagus nerves. Windle further noted experimental species differences and the decline in heart rate as a consequence of asphyxia following bilateral vagotomy (see Young, Sealy, Harris and Botwin, '51). Kellogg ('27) was unable to inhibit the hearts of several different mammals at term by stimulating the vagus nerves though there was some indication that the postganglionic mechanism in the heart was functional.

Other investigators have reported that variable amounts of vagus influence were present at birth, or early in postnatal life. Thus, the observations in cat and dog fetuses after experimental Caesarean section, and in the young after birth, led Clark ('32, '34) to the conclusion that pressor reflexes make their appearance about three to 4 days after birth. As a result of faradic stimulation of the depressor and carotid sinus nerves, Bauer ('38) was able to demonstrate some reflex inhibition of the rabbit heart 11 to 14 days after birth.

Several investigators have noted further that atropine had little or no effect upon the heart rate of newborn animals (Anrep, 1880; Lhota, '11; Scott and Truex [unpublished data]). Anrep (1880) believed also that the decrease in heart rate which accompanied an increase in age and body weight, was due to decreased automaticity of the sinoatrial node and an increase in vagal tone.

It is of interest to note that two other human functions, related embryologically and physiologically to the vagus nerves, appear to be immature at birth. Miller, Proud and Behrle ('52) obtained a cough reflex in only 25% of their human infant series during the first 5 days of life. However, in infants over one month of age the cough reflex was present in 90%. Inasmuch as the gag and swallow reflexes were present in all but two of 154 infants, these authors believed the absence of the cough reflex was due to immaturity of the vagal afferent fibers. In a similar way a minimal amount of vagal influence on the gastrointestinal tract at birth was indi-

cated by its reduced peristaltic activity (Ivy, '48), and the low output of hydrochloric acid by the stomach (Cutler, '38).

Adelson ('53) recently called attention to the importance of cardiac syncope of neurogenic origin and stated, "there is no particular age or sex distribution for death from (vagal) inhibition."

The present experimental studies were undertaken to determine both the anatomic development and the physiologic effects of vagal stimulation in a young laboratory animal of known age and weight. It was the feeling of the authors that such a combined attack upon the problem would provide needed quantitative information to either substantiate or invalidate the loosely used term "vagal tonus."

MATERIALS AND METHODS

In the course of this investigation over 200 puppies of known age were used for either acute or chronic experiments and histologic studies.

The standardized procedure itemized in figure 1 was uniformly followed in 112 animals and forms the basis for this communication. As indicated in this figure the puppies ranged from one to 73 days of age and represented litters from all sizes and breeds of dogs. At the time of the experiment the animals were weighed and an electrocardiogram was taken on an Edin direct-writer electrocardiograph before anesthesia was administered. All ECG records and RR measurements were made from lead II. The animals were then anesthetized by intraperitoneal Nembutal® with a dosage of 35 mg per kilogram of body weight, and upon relaxation an ECG record was made.

At this point mid-cervical skin incisions were made and both the left and right vagus nerves were gently freed from the carotid sheath and artery for a distance of two inches. Double cotton ligatures were passed below the exposed nerves, and with a continuous ECG record being taken, either the left or right nerve was tied, cut, and the distal stump stimulated. A platinum point shielded electrode was applied to

the nerve and all stimulations were made with an Electrodyne stimulator at 20 volts and a frequency of 30 per second. The heart was allowed to recover to provide a record of the heart rate after unilateral vagotomy. A similar procedure was followed while the second nerve was tied, cut, and stimulated as indicated in figure 1.

Each experiment took but a few minutes and at the conclusion each animal was sacrificed. The hearts were removed immediately and uniformly trimmed and weighed. Weights and electrocardiographic data obtained during these experiments are indicated to the right of figure 1. After final com-

PROCEDURE AND DATA	GRAPH
112 PUPPIES WEIGHED (1-73 DAYS OF AGE)	— BODY WEIGHT
UNANESTHETIZED ECG RECORD (LEAD II)	— NORMAL HEART RATE
ECG AFTER ANESTHESIA (I-P NEMBUTAL $\frac{35 \text{ mg}}{\text{KILO BODY WT.}}$)	— ANESTHESIA HEART RATE
1 st VAGUS NERVE EXPOSED AND TIED WITH ECG RECORD	— 1 st TIE HEART RATE
1 st NERVE CUT AND DISTAL STUMP STIMULATED ($\frac{20 \text{ v}}{30 \text{ f}}$)	— 1 st STIMULATION HEART RATE
POST-STIMULATION RECOVERY AND ECG	— UNILATERAL VAGOTOMY RATE
2 nd VAGUS NERVE EXPOSED AND TIED WITH ECG RECORD	— 2 nd TIE HEART RATE
2 nd NERVE CUT, RECOVERY WITH ECG RECORD	— BILATERAL VAGOTOMY RATE
2 nd NERVE DISTAL STUMP STIMULATED	— 2 nd STIMULATION HEART RATE
ANIMAL SACRIFICED, HEARTS TRIMMED AND WEIGHED	— HEART WEIGHT

Fig. 1 Sequence of experimental procedure followed in vagal stimulation. Electrocardiographic (ECG) records used to construct the subsequent graphs are indicated on the right.

pilation the RR of the ECG record was converted to heart rate per minute (Ashman and Hull, '41) in order to simplify interpretation of the graphs.

The hearts of 20 puppies and two adult dogs were used for microscopic study of the atrial neural elements. The puppy specimens (one to 200 days of age) were either perfused in vivo, or placed in appropriate fixative solutions immediately after removal. Fixation in alcoholic chloral hydrate, ammoniated absolute alcohol or 10% neutral formaldehyde was followed by the routine silver procedures of the Cajal, Ranson, and Bódián techniques. Some specimens of different age groups were fixed in a solution of saturated mercuric chloride and 10% neutral formaldehyde. These, as well as

some formaldehyde fixed tissues, were prepared for study of the Nissl material by the Gallocyanin method. All of the microscopic serial sections were cut from paraffin blocks at thicknesses of 8 to 12 μ .

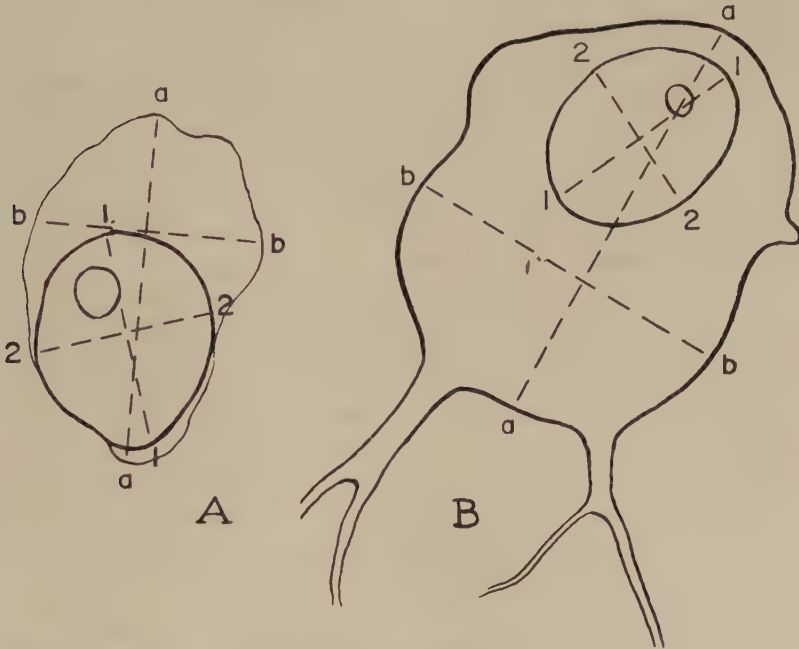


Fig. 2 Diagram to indicate method of measuring immature (A) and mature (B) nerve cells of the intrinsic atrial ganglia. The long (a-a,) and short (b-b,) diameters of the cytoplasm were measured as well as the similar diameters of the nucleus (1-1, 2-2). Results of such measurements are shown in figure 7.

With the aid of an ocular micrometer and an oil immersion objective, two separate measurements were made of both the nuclear and cytoplasmic diameters in each ganglion cell as indicated in figure 2. A total of 100 ganglion cells in the sinoatrial node area was measured in each of 22 specimens.

RESULTS

Age, weight, heart rate, and anesthesia

It was not too surprising to find considerable variation in size of puppies on the day of their birth, for as noted above

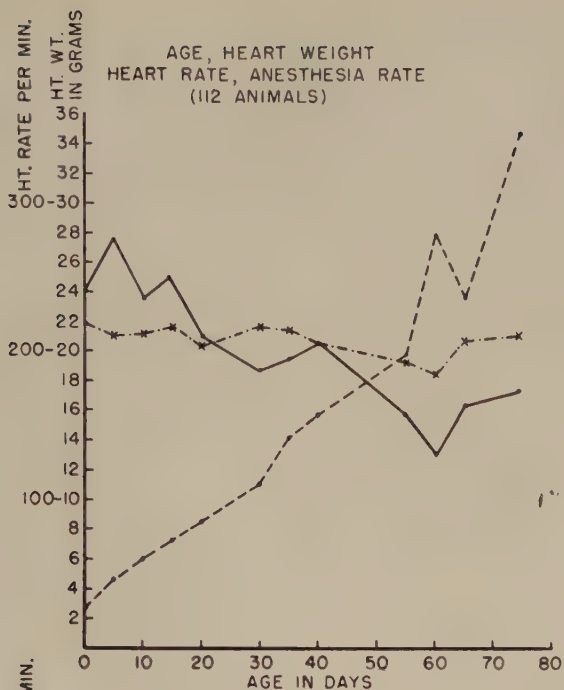
our animals were the progeny of a heterogeneous parentage. Such variance is largely responsible for the irregularity of the heart rate statistics presented in figure 3A.

It was evident that as the animal became older the heart weight increased, whereas the rate of the heart decreased (fig. 3A). However, we were surprised to note that the Nembutal anesthesia used in these experiments demonstrated a marked slowing effect on the heart rate in the first 20 days after birth. In animals over 20 days of age the heart rate of the anesthetized animal was consistently more rapid than the pre-anesthetized heart rate. Thus, Nembutal appeared to block some of the vagal inhibition of the heart (i.e. the heart was released from vagus influence) in animals over 20 days of age. While the action of this drug was known, its significance in relation to this series of experiments became apparent only as we progressed. We found it virtually impossible to obtain accurate heart rates on active unanesthetized puppies, but RR intervals recorded from anesthetized animals yielded a more consistent *actual* heart rate on which comparison could be based. Hence, in subsequent graphs the control rate for each animal is that taken while under anesthesia and for the sake of brevity is referred to herein as the control rate.

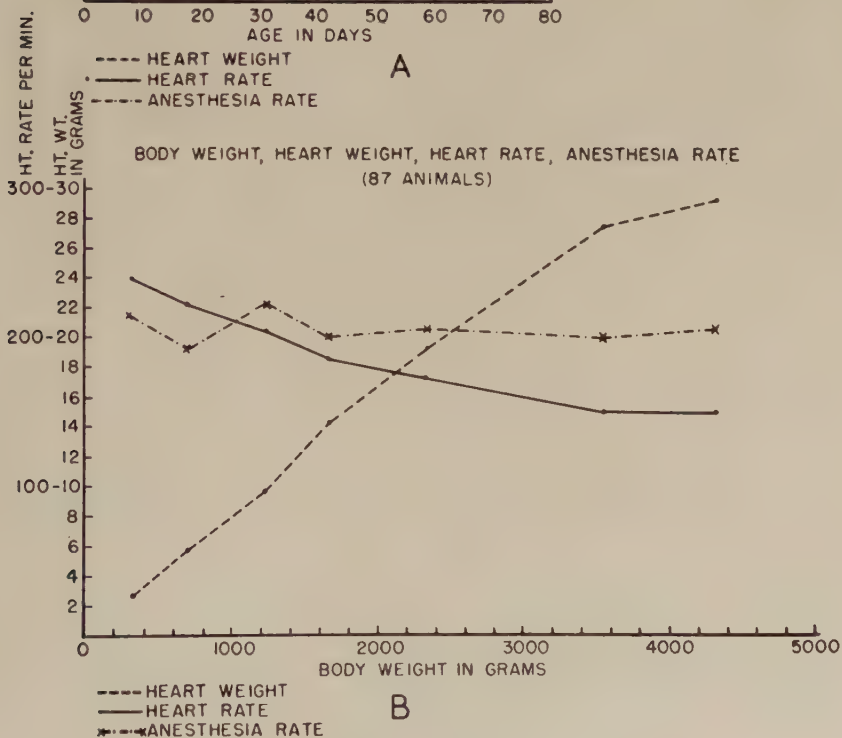
The authors were interested in demonstrating a better graphic correlation between heart weight and heart rate than that depicted in figure 3A. For this reason 87 of the 112 animals were selected solely on the basis of their individual body weight. The data for such animals was then compiled and is shown in figure 3B. It is evident that body weight, heart weight, and heart rate provide better indices of correlation (fig. 3B) than do age, heart weight, and heart rate (fig. 3A). Again one can see that the anesthesia caused a slowing of the rate of the hearts of animals weighing less than 1000

Fig. 3A Graph of heart weight and heart rate of 112 animals plotted in accordance to their postnatal chronological ages. The heart rate is indicated before and after anesthesia in this animal series.

Fig. 3B Graph of heart weight and heart rate of 87 selected animals (of the 112 animals in A above) plotted against their body weights.



A



B

Figure 3A and 3B

gm, whereas anesthesia accelerated the heart rate in animals with a body weight in excess of 1000 gm (fig. 3B). However, figure 3B shows a reasonably stable rate that approximated 200 beats per minute in the anesthetized animal regardless of age or body weight.

Effect of tying vagus nerves on heart rate

At the instant either of the vagus nerves were tied the ECG record invariably demonstrated a temporary decrease in the heart rate which persisted for one or two beats. The

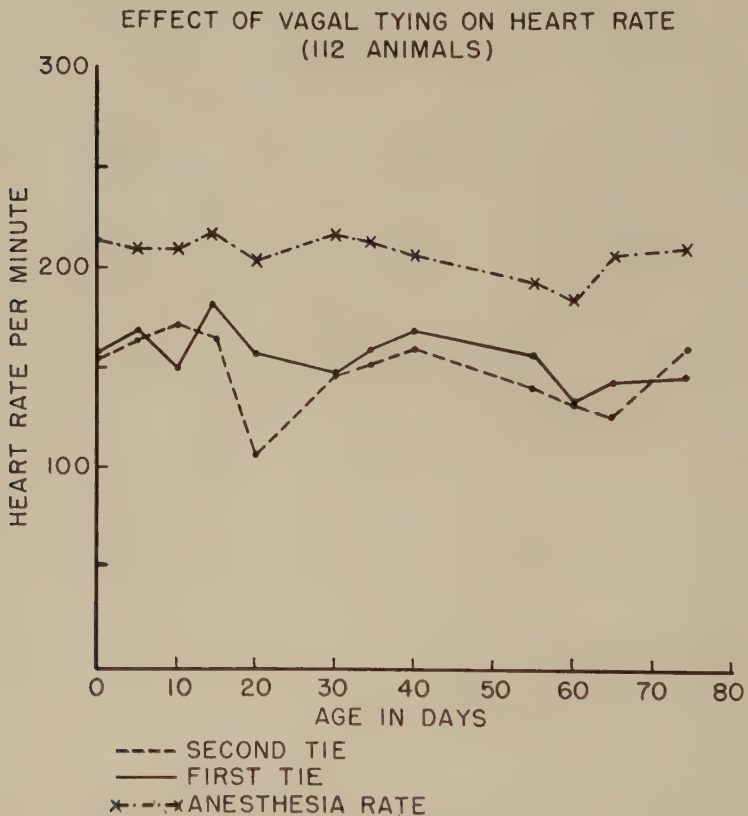


Fig. 4 Graph indicating the temporary decrease in heart rate induced by mechanical act of tying one, then both vagus nerves. In all subsequent graphs the heart rate under anesthesia served as the control.

mean decrease in heart rate in animals of different ages that was induced by the mechanical act of tying is shown in figure 4. It was evident that the vagus nerves could be stimulated by such a mechanical act and influence the heart rate in animals of all ages. However, we did not observe marked slowing of heart rate or complete inhibition in any of the animals as a result of tying one or both vagus nerves. The heart rate decreased more after the second nerve was tied, but the difference in rates after the first and second ligatures was not significant. The tying of the right vagus nerve usually induced a greater decrease in heart rate than did tying of the left vagus nerve. This dominance of right vagus inhibition was observed regardless of whether this nerve was tied first, or after the left vagus nerve was tied. After the brief decrease in rate shown in figure 4, the cardiac rate rapidly returned to the control rate.

Effect of vagal stimulation on heart rate

Stimulation of either vagus nerve in the cervical region induced a slowing of the heart rate in animals of all ages (fig. 5). The *left* nerve was tied, cut and stimulated first, and the right nerve immediately thereafter in one-half of the experimental animals. In the remaining half of the animals the order of stimulation was reversed. To facilitate a comparison of these stimulations the results are shown in two graphs (figs. 5A and 5B).

The left vagus nerve, when stimulated first, exhibited a variable amount of influence upon the heart rate as shown by the solid line in figure 5B. Although such stimulation decreased the heart rate, subsequent stimulation of the right vagus nerve always induced a greater decrease in rate. If, on the other hand, the right vagus nerve was stimulated first and the left nerve second (fig. 5A), the right vagus nerve again demonstrated a greater influence on cardiac rate. Thus, the right vagus nerve seemed to be capable of uniformly exerting more inhibition on cardiac activity than the left vagus, within the 73 day postnatal period studied.

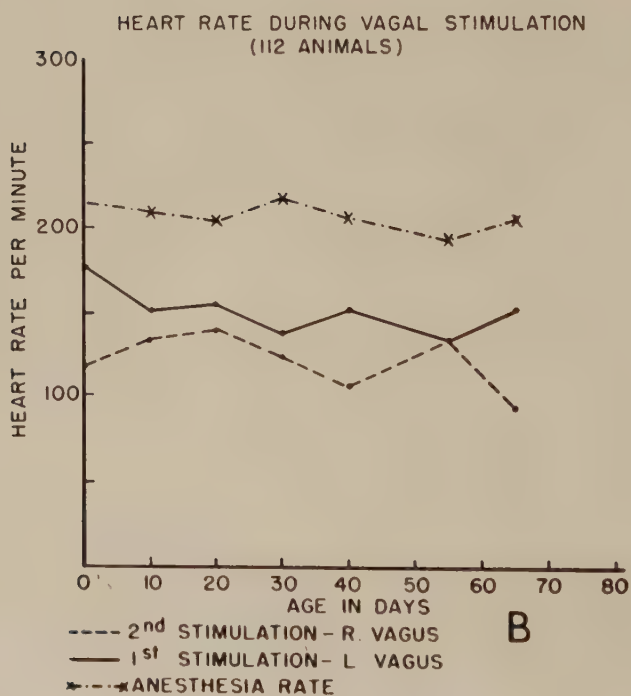
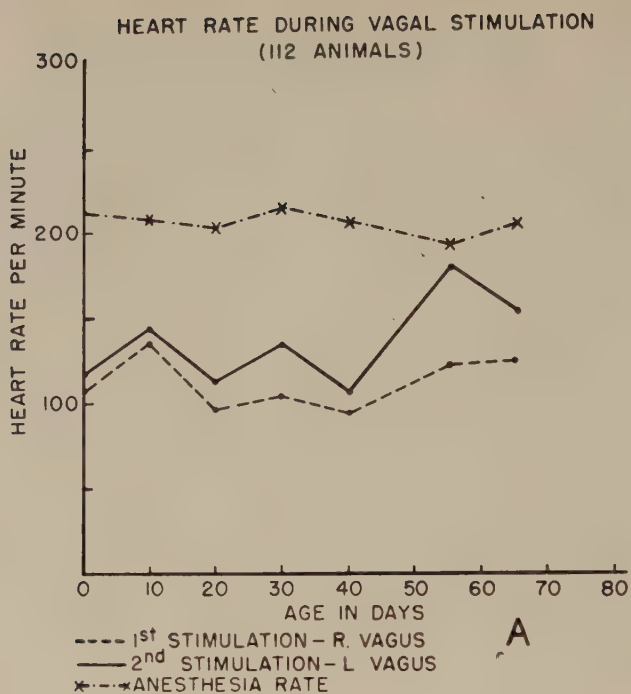


Figure 5A and 5B

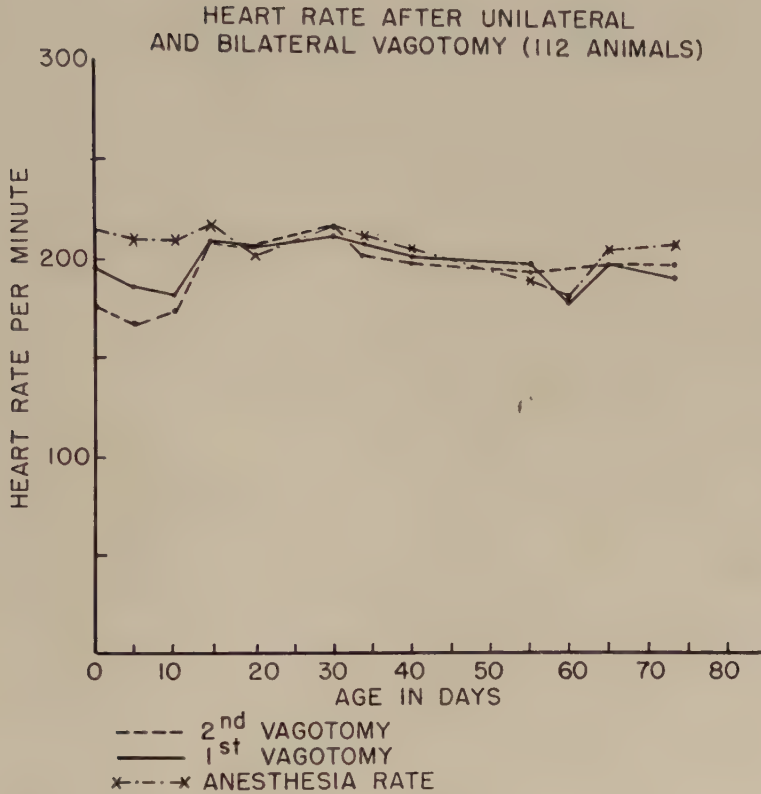


Fig. 6 Graph to demonstrate heart rate following resection of one or both vagus nerves. The rate increased after resection and stimulation to approximate the anesthetic rate.

*Effect of unilateral and bilateral vagotomy
on heart rate*

In these experiments a continuous ECG was taken for several minutes after unilateral and bilateral vagotomy to determine the extent of cardiac recovery. The results are shown in the graph of figure 6.

Fig. 5 Graph indicating decrease in heart rate induced by stimulation of vagus nerves at different ages. In A the right vagus nerve was stimulated first, and the left nerve second. In B the left vagus was stimulated first, and the right nerve second. Note the greater decrease in rate during right vagus stimulation.

In animals under two weeks of age the stimulation of the distal stump of one or both vagus nerves caused a decrease in heart rate, so that the latter did not return to the control rate within the time interval of our experiments (fig. 6). However, in the animals over 20 days of age the mean recovery heart rate increased after unilateral and bilateral vagotomy to again approximate the pre-operative control heart rate. In a few instances the recovery rate, after vagotomy, exceeded the control heart rate, and then by only a few beats per minute. Thus, upon stimulation both vagus nerves could decrease heart rate, yet when the influence of these same nerves was totally removed, the heart failed to beat any faster than it did when they were intact.

Ganglion cells. Location and structure

Study of serial sections of the sino-atrial node area revealed an abundance of ganglion cells from the day of birth onward. The clusters of ganglia were usually located in the epicardium, although they were observed frequently as scattered neural elements among the cardiac fibers of the atrial myocardium or adjacent to the node itself. As noted by several previous investigators (Bachman, '23; Nomura, '51; Davis, Francis and King, '51; Glomset and Cross, '52), the larger ganglia were most numerous in the interatrial and atrioventricular sulci, and within the sulcus terminalis. Other ganglionic masses generally were observed between the aorta and pulmonary artery and on the dorsal and right lateral margins of the superior vena cava.

Microscopic examination of the ganglion cells within the hearts of animals of various ages revealed striking differences. The intercardiac neurons appeared to be immature at birth as indicated by the several staining procedures employed. When the two diameters of the nucleus and cytoplasm were measured (fig. 2) the average nucleus-cytoplasm ratio ranged from 1.43 at one day of age to 1.80 at 20 days. In animals 100 days of age or older, the average ratio was two. The mean

data obtained by measuring 100 ganglion cells in the sinoatrial nodal area of the 22 animals is presented graphically in figure 7.

The undifferentiated ganglion cells shown in figures 8, 9, and 11 account for the low nucleus-cytoplasm ratio we obtained in our young animals (fig. 7). Such neurons have large nuclei, distinct nuclear membranes and scanty amounts of cytoplasm

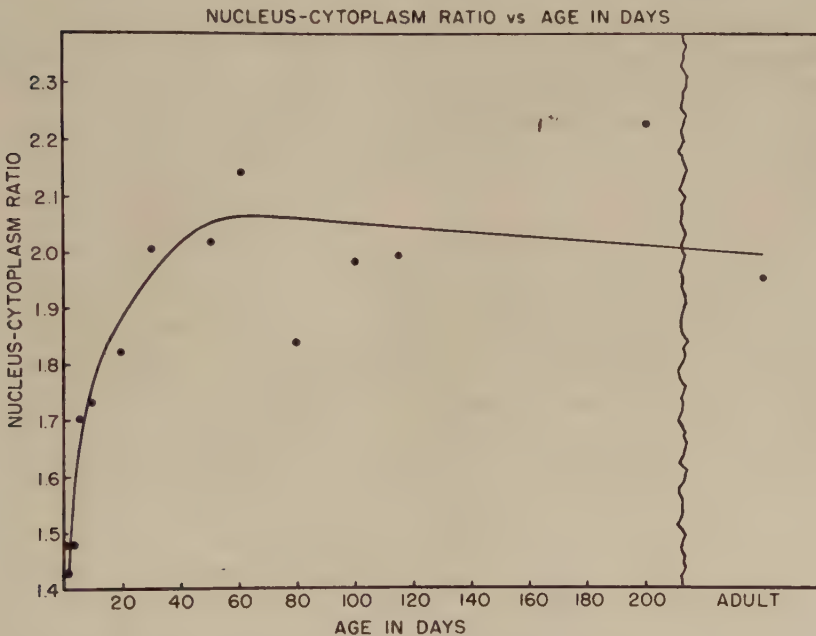


Fig. 7 Graph of nucleus-cytoplasm ratio in 22 animals. Note small ratio indicating immaturity between birth and 40 days of age.

devoid of neurofibrillae and Nissl material (figs. 8 and 9). Most of the intracardiac ganglion cells also lack stainable dendritic and axonic processes (fig. 8). However, in the atrial ganglia of even the youngest animals, one sees an occasional neuron which is conspicuous by its size, processes and darker stain (arrow in fig. 11). Such differentiated ganglion cells possess discrete postganglionic processes that can be traced to very fine terminal branches in the adjacent myocardium.

Comparative studies showed that the cell argyrophilia increased between birth and 60 days of age. At the latter stage most of the silver-stained ganglion cells had neurofibrillae and one or more processes. The mature appearance of the ganglion cells is particularly well shown in figure 12. The right vagus nerve had been removed in this animal at 30 days of age, and the paucity of preganglionic nerve fibers enabled one to better see the processes of the individual cells.

Although there was little or no Nissl material stained in the cardiac ganglion cells at birth (fig. 9), small amounts of this material usually were present by 80 days of age. By 100 days of age the pattern of the Nissl material was similar to that observed in the cardiac ganglion cells of an adult animal (fig. 10).

Nerve fibers

Extensive study of silver-stained serial sections by one of the authors (D.M.L.) revealed numerous fine nerve fibers in the atrial myocardium, sinoatrial node and coronary arteries at birth. A few fibers appeared to be postganglionic processes from the intracardiac ganglia, whereas most of the tortuous, branching fibers did not appear to arise from such ganglia and were presumed to be of sympathetic origin.

The course and branchings of the nerves in three serial sections were traced and are shown in a composite drawing (fig. 13). The fine, beaded, branching nerve fibers could be followed for considerable distances as they coursed through the myocardium. No specialized nerve endings were observed in our preparations. The fibers often terminated in relation to the nucleated area of a cardiac muscle fiber (1 in fig. 13), or ended abruptly between the fibers (2 in fig. 13). The characteristic beaded appearance and clubbed, epilemmal terminals of the fine myocardial nerve fibers are shown under higher magnification in the insert of figure 13. It was not uncommon to find a network, or neurofibrillar enlargement, near the point where nerve branching occurred. Such expanded networks often had several fine branches arising from them. The finer

filaments seemed to lose their staining affinity and disappeared in the section (1 in insert of fig. 13).

It seemed that our specimens demonstrated "areas of nerves" interspersed with regions in which there were very few nerve fibers. We observed such heavy and light areas of innervation in hearts that had been stained by several neurological techniques, i.e. bulk silver and other staining methods. For this reason we did not believe such areas were the result of faulty impregnation. It should be pointed out that, as the hearts of older animals were studied, the neural areas were more frequent, and the concentration of nerve fibers within them increased. The larger and medium-sized nerve fibers were distributed as a myocardial plexus. Finer branches of the plexus approached the endocardial surface and ended as small terminal clubs.

In the 11 younger specimens of our series the sinoatrial node (one to 20 days of age), had fewer neural elements than the adjacent atrial myocardium. In two specimens 60 and 200 days of age, no difference was observed in the number of nerve fibers in the sinoatrial node and the surrounding atrial musculature.

In addition to the areas of myocardial innervation, we observed numerous fine nerve fibers forming a plexus about the coronary arteries and their branches (fig. 14). The larger nerve fascicles were located in the tunica adventitia. Branches issued from the larger nerve bundles and often formed a rich anastomotic network in this outer tunic (1 in fig. 14), while fine rami seemed to penetrate the outer limits of the tunica media to terminate as small knobs or naked endings between the smooth muscle fibers. It was not uncommon to observe expansions along the course of a nerve fiber such as that shown at 2 in figure 14. This expanded portion of the fiber appears to be a network composed of very fine neurofibrillae, similar to that described by Polley ('55) in the walls of the peripheral arteries of the cat prior to sympathectomy.

In animal hearts of all ages the innervation of the coronary arteries was profuse. No differences were observed between

the arterial innervation of newborn puppies and that of young adult dogs.

DISCUSSION

Although the preganglionic fibers of the vagus nerves had reached the heart of the newborn puppy, the postganglionic cells were undifferentiated and lacked stainable processes. As pointed out above, these intrinsic cells have a low nucleus-cytoplasm ratio and are devoid of neurofibrillae and Nissl substance. The notable exception was the occasional differentiated neuron such as that indicated by the arrow in figure 11.

It is evident that the few differentiated intracardiac ganglion cells could explain the decrease in heart rate that resulted from physiological stimulation of the vagus nerves in the newborn dog. Such scattered neural elements could also account for the temporary slowing that accompanied the mechanical tying of these nerves.

We realize that in our experiments the vagi were stimulated by a supramaximal voltage that far exceeded normal physiologic conditions of the intact animal. In each experiment we believe we delivered an adequate stimulus. We reiterate that in no instance did we produce cardiac inhibition in puppies following elevation of either the voltage or the frequency. However, in our previous studies (Hamaty and Truex, '54) we were able to inhibit the hearts of adult dogs for prolonged periods with the same voltage and frequency used in the present experiments. The right vagus nerve had more influence than the left vagus upon the activity of the sinoatrial node of the adult dog.

Our experiments indicate that a similar dominance is present in the newborn under physiologic conditions. We cannot say at the present time, just how much inhibition these two nerves normally exercise upon the newborn heart. They probably exert only a minimal influence in the first few weeks after birth, for the heart rate seldom exceeded the control rate after both vagus nerves were severed in the neck (fig. 6). It is doubtful whether the rate would have increased if our vagotomized animals had been followed for longer post-opera-

tive periods. The paralysis of the vocal cords and respiratory embarrassment often leads to early post-operative dyspnea and anoxia; a factor we were mindful of while interpreting our physiologic data. We cannot explain the decreased heart rate in anesthetized animals under 1000 gm body weight. Whether the postganglionic sympathetic nerve fibers, or the intrinsic cardiac muscle fibers are more sensitive to Nembutal in this early postnatal period remains to be determined, as do the discrepancies between our physiologic data and the anatomic information of the neural elements in animals older than 60 days of age. The intrinsic ganglion cells were approaching structural maturity, yet vagus stimulation resulted in ECG records that indicated immaturity. It is obvious that additional experiments on older animals are required if we are to determine the precise time when the vagus nerves assume their full inhibitory influence on the heart. Such investigations are planned by the present authors.

In view of the above neural immaturity and minimal amount of physiological activity by the vagus nerves, one must conclude that another factor was responsible for most of the decrease in post-natal heart rate. If one re-examines the data in figure 3B the close correlation between body weight, heart weight, and heart rate now assumes greater significance. It is evident that larger hearts (i.e. with a high body weight-heart weight index) beat slower; a point that was made by Clark ('27). Our results substantiate his conclusions. We regret that we did not include basal metabolic rate studies on our experimental animals to determine whether such rates decreased along with heart rates.

The fine myocardial and vascular nerves that are so evident at birth were presumed to be of sympathetic origin in view of the immature cardiac ganglion cells. Whether the sympathetic neural elements reach the heart prior to the vagal fibers is a question that must also await further anatomic investigation. Kuntz ('53) has stated that the sympathetic nerves grow into the cardiac and pulmonary plexuses, but not until the primordia of the ganglia of these plexuses are well estab-

lished. The complex intermingling within the heart of sympathetic, parasympathetic and sensory nerve fibers makes the isolation of each of the different fibers an almost impossible task by our existing anatomic methods. For example, our attempts to produce transneuronal degeneration in the intrinsic cardiac ganglion cells were negative. We observed no consistent changes in the peripheral postganglionic cells of either the newborn or adult dog hearts 6, 8, 10 or 12 months after left or right unilateral cervical vagotomy (Shull and Truex, unpublished data). The intrinsic ganglia and their processes within the heart defy complete elimination in newborn puppies and adult dogs. Although it is impossible to provide quantitative evidence of the precise number and distribution of the different types of nerve fibers within the heart, one can gain considerable information about such nerve fibers following selective surgical resection of the different extrinsic neural components. The initial extirpation studies of Wool-lard ('26) and Nettleship ('36) on the afferent cardiac innervation have indicated the feasibility of such an approach. Combinations of sympathectomy, vagotomy, and dorsal root ganglionectomy have already been undertaken in our laboratories. It is hoped that both a physiologic and anatomic study of these experimental animals will provide information relative to some of the above unanswered questions.

Our observations in the newborn dog cannot be inferred as applicable in the human newborn infant, since our experimental animal has a gestation period of only 60-63 days. However, other investigators have noted evidence of vagal immaturity at birth in infants (Ivy, '48; Cutler, '38; Miller, Proud and Behrle, '52). In a similar way other portions of the human nervous system are known to be immature at birth (e.g. pelvic autonomies, corticospinal pathways, visual system, etc.). It is regrettable that so little is known of the initiation of the carotid sinus reflex in human infants, for this mechanism was believed a possible cause of neurogenic syncope and death in the newborn (Adelson, '53).

SUMMARY

This paper is based upon data obtained by (1) the electrical stimulation of both vagus nerves in 112 puppies (one to 73 days of age) to determine the effect of stimulation upon the electrocardiographic heart rate; and (2) a histologic study of the intrinsic atrial neural elements in 22 additional animals (one day of age to maturity).

Both vagus nerves were capable of slowing the heart rate from birth onward, but never induced complete cardiac inhibition. A temporary decrease resulted from the mechanical tying of the nerves, whereas electrical stimulation of the severed distal nerve stump produced a sustained reduction in heart rate. In our experiments the right vagus nerve produced a greater decrease in heart rate than tying and stimulation of the left vagus nerve. In animals 20 days of age or older, the heart rate increased to approximate the control rate following unilateral and bilateral vagotomy. We concluded that there was a minimal amount of vagal inhibition upon the heart of young dogs within the 73 day period studied.

The majority of the intrinsic atrial ganglion cells were structurally immature at birth, as indicated by their low nucleus-cytoplasm ratio; absence of neurofibrillae, stainable cell processes and cytoplasmic Nissl material. Neurofibrillae and cell processes were present at 60 days of age, while the Nissl material attained an adult pattern by 100 days of age. A few scattered atrial ganglion cells were structurally mature at birth. We believe the latter postganglionic neurons account for the decrease in heart rate observed following experimental vagal stimulation. The increase in body weight and heart weight were correlated closely with the decrease in heart rate in our animal series.

Study of silver-stained serial sections revealed numerous fine nerve fibers within the atrial myocardium and along the coronary vessels. No specialized or hypolemmal nerve endings were observed. Our studies indicated that the postganglionic sympathetic fibers reached these cardiac structures before the postganglionic parasympathetic neural elements were

fully developed. The significance of these results in the new-born animal are discussed.

ACKNOWLEDGMENTS

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We express our thanks to Miss Edna McCrane and Miss Donna Lee Stringfellow for their excellent technical and secretarial assistance.

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PLATE 1

EXPLANATION OF FIGURES

- 8 Atrial ganglion cells in the heart of a one day old puppy. Note the large nuclei (arrows), scanty cytoplasm, and absence of neurofibrillae and cell processes. Bodian protargol technic. $\times 520$.
- 9 Atrial ganglion cells in the heart of a one day old puppy. Note large nuclei (arrows) and absence of Nissl material in the cytoplasm. Compare with figure 10. Galloeyanin technic. $\times 520$.
- 10 Atrial ganglion cells in the heart of an adult dog. Note pattern and appearance of Nissl material. Galloeyanin technic. $\times 250$.
- 11 Atrial ganglion cells in the heart of a two day old puppy. Most of the cells above the center are immature. Note the large, mature ganglion cell at the bottom of the figure (arrow). Ranson silver technic. $\times 570$.
- 12 Atrial ganglion cells in the heart of a 60 day old puppy. Note neurofibrillae in the abundant cytoplasm. The cell processes are clearly seen, as the pre-ganglionic fibers of the right vagus nerve were removed at 30 days of age. Ranson silver technic. $\times 470$.

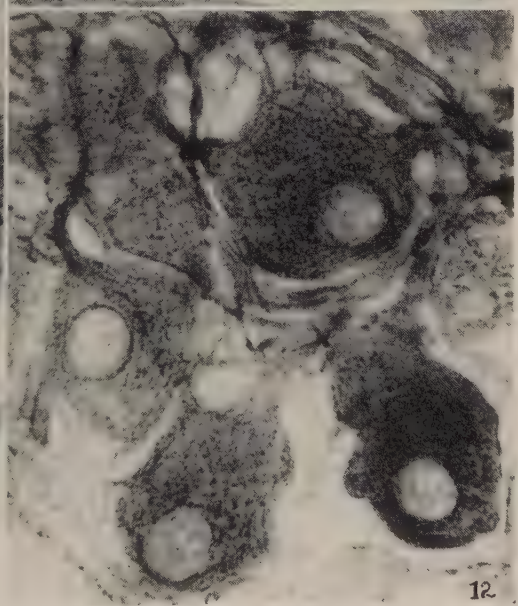
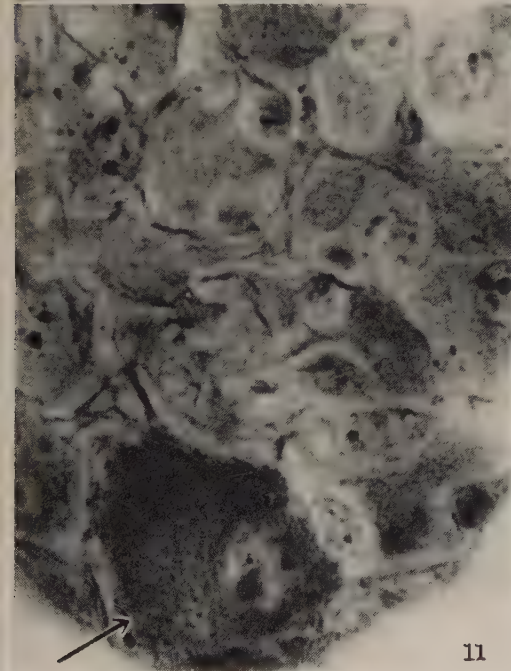
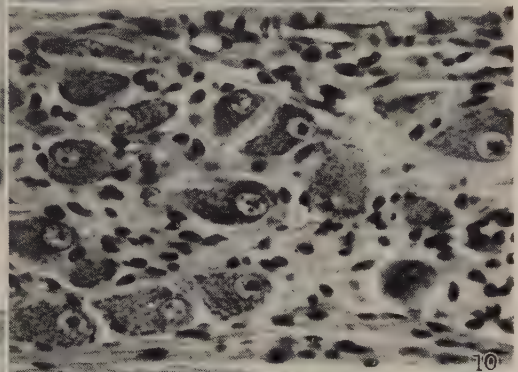
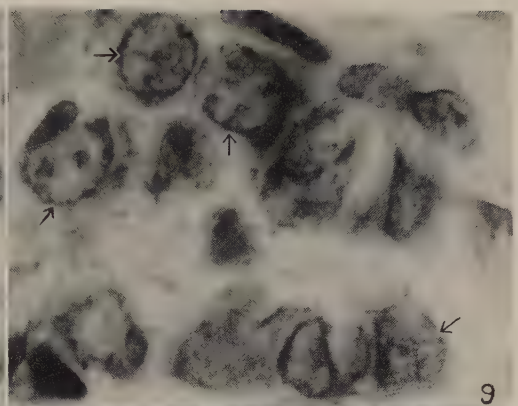
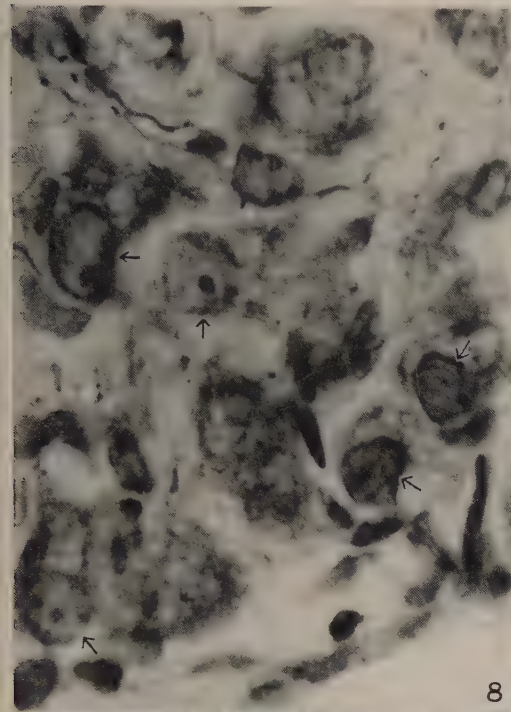
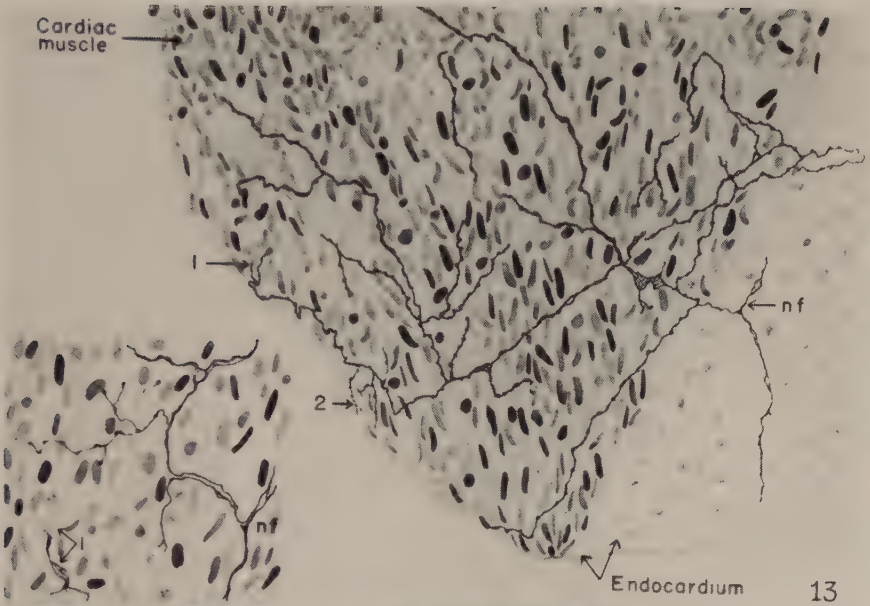


PLATE 2

EXPLANATION OF FIGURES

- 13 Myocardial nerve fibers in the right atrium of a 7 day old puppy. The nerve fibers (nf) terminate as simple knobbed endings in relation to the nucleated portion of the cardiac muscle fiber (1), or end between the muscle fibers (2). The insert drawing to the left, shows the finest nerve fibers at a higher magnification ($\times 725$). The double arrow (1) indicates a small neurofibrillar expansion with terminal filaments that were lost within the section. Holme's silver technic. $\times 375$.
- 14 Nerve plexus along a small branch of the right coronary artery (A). B is a smaller branch of the parent vessel. Note the larger nerve bundles (nf) and network (1) of fibers within the tunica adventitia. Some of the nerve fibers demonstrate larger neurofibrillar expansions at 2. Heart of 7 day old puppy. Holme's silver technic. $\times 375$.



HISTOLOGICAL STUDIES OF THE ADRENAL GLANDS AFTER REPEATED INJECTIONS OF HISTAMINE IN ALBINO MICE¹

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SIX FIGURES

Histological studies by Sheusi ('51) indicated that a single subcutaneous injection of histamine in the mouse produced both an immediate local, and a prolonged systemic, reaction. Changes in the fibrous connective tissues of the body accompanied changes in the adrenal glands and both were part of the systemic reaction. The purpose of the present studies was to investigate further the nature of the changes in the adrenal glands not only after a single injection but especially following repeated subcutaneous injections of histamine.

MATERIAL AND METHODS

Experiment 1

Eleven male albino mice were injected subcutaneously, once a day, with 0.1 ml of a 1:500 dilution of histamine acid phosphate in sterile physiological salt solution. One mouse was sacrificed each day within an hour after the injection, hence the last mouse of the series received 11 injections. Intact, untreated mice served as controls. The adrenal glands of each mouse were fixed in formol-saline and embedded in paraffin. Sections were stained with hematoxylin and eosin, and by the periodic acid-Schiff technic. The latter is a useful method

¹This work was aided in part by a grant from the Banting Research Foundation and by a Canadian National Health grant.

for demonstrating changes in connective tissue fibers, basement membranes, and some unidentified secretory products of the adrenal glands.

Experiment 2

After the histological study of the tissues from this series had been made, an additional 48 male albino mice were injected subcutaneously, once a day for 8 days with the same dilution of histamine acid phosphate as in experiment 1. A blood smear and differential blood count were made on each mouse every day immediately following the injection of histamine. The animals were sacrificed on the 8th day, after the final injection and the adrenal glands were prepared for histological study as in experiment 1.

OBSERVATIONS

Experiment 1

The microscopic appearance of the adrenal glands removed after one subcutaneous injection of histamine differed from that of the control (fig. 2) only slightly. The medullary cells were more granular, and the zona glomerulosa cells showed more mitotic figures and contained more lipid droplets than in the control.

In the adrenal glands from the mouse that was injected on two consecutive days with histamine the following changes were noted (fig. 3). The medullary cells were enlarged, elongated, and some contained vacuoles. The cells of the glomerular and transitional zones of the cortex were hypertrophied, elongated and contained lipid droplets. The cortical capillaries were somewhat distended. At the cortico-medullary junction the reticular fibers were more intensely stained with the Schiff reagent than in the control.

In the adrenal glands from the mouse that was injected on three consecutive days, additional changes were observed (fig. 4). The medullary cells were hypertrophied, granular and more vacuolated. The cortex was somewhat thicker. The

cells of the glomerular and transitional zones were moderately large, elongated and contained moderate amounts of lipid droplets. Mitotic figures occurred in the transitional zone and in the zona fasciculata. The latter was thickened and its cells contained many lipid droplets. Large and small Schiff-positive cells were found in the zona reticularis. Some of these were vascular reticulo-endothelial phagocytes, the others, parenchymal cells of this zone. The reticular fibers at the cortico-medullary junction were intensely stained with the Schiff reagent, in contrast with the control.

After the 4th and 5th injections the basement membranes of the capillaries and sinusoids were thicker and the cortical capillaries were more distended. After the 6th injection (fig. 5) many of the medullary cells were hydropic. Here and there in the medullary sinusoids, lymphopoietic foci were seen. The capsule of the adrenal was thickened, the cortical capillaries were more distended and their basement membranes were thickened and stained with the Schiff reagent. Although the cortex was still thickened and many mitotic figures were found in the glomerular and transitional zones, the cells of the zona fasciculata were smaller and few contained lipid droplets. Many of the cells in the zona reticularis contained vacuoles of various sizes. Schiff-positive macrophages were found in both the cortex and medulla.

Following each subsequent injection of histamine from the 7th to the 11th day (fig. 6) the medullary cells were increasingly hydropic. Lymphopoietic foci in the medullary sinusoids occurred more frequently. In the glomerular and transitional zones of the cortex, mitotic figures occurred and many of the cells of these zones contained lipid droplets. The thickness of the cortex was considerably reduced by the atrophy of the cells of zona fasciculata and a reduction in size of the cells of zona reticularis. The capsule and the capillary basement membranes were increasingly thicker. Schiff-positive macrophages and parenchymal cells were increased in number in the cortex, medulla and cortico-medullary junction.

Experiment 2

From the series of 48 mice given 8 daily subcutaneous injections of histamine, only 93 adrenal glands were available for microscopic study due to the loss of three adrenal glands during the process of preparation of the material for histological sections. Several sections from each of the 93 glands were studied under the oil immersion lens. The degree of hypertrophy and of hydropic changes in the medullary cells was estimated on the basis of the size of the cells, the size and staining of the nuclei, the number and size of the vacuoles, and the number of cells exhibiting these changes, as compared with the control. The degree of these changes was recorded as none (fig. 2), minimal (fig. 3), moderate (fig. 4), marked (fig. 5) and maximal (fig. 6). The degree of atrophy of the zona fasciculata was estimated on the basis of the amount of lipoid droplets remaining in the cells of this zone, as compared with the control, and recorded in the same way.

As might be expected, there was considerable variation in the microscopic appearance of these adrenal glands. However, when distribution curves of the number of adrenals exhibiting the degree of change in each category were made, it was found that 73 of the glands showed marked or maximal hypertrophy of the medullary cells. Only one gland showed minimal hypertrophy of these cells. Hydropic changes in the medullary cells were marked or maximal in 63 glands and only one gland showed no hydropic change. In several of the glands mitotic figures were found in the medullary cells.

It was difficult to assess the degree of atrophy of the zona fasciculata because there was great variation in the extent of the glomerular and transitional zones and in the content of lipoid droplets within the cells of these zones. However, in 60 of the glands the cells of the zona fasciculata contained no, or minimal amounts of, lipoid droplets. In only one adrenal did the cortex contain the maximum amount of lipoid droplets. Only one mitotic figure in a parenchymal cell of the

cortex was found in all the sections studied and that was in the zona glomerulosa.

The daily differential blood counts also showed considerable variation. The percentages of eosinophiles, neutrophils and non-granular leucocytes were plotted on scatter graphs.

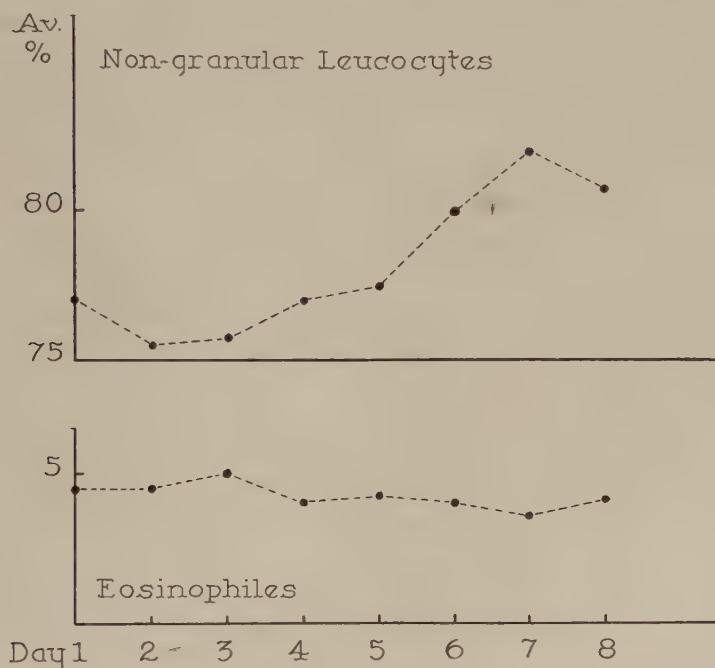


Fig. 1 The average of percentages of non-granular and eosinophile leucocytes from differential blood counts of 48 mice injected subcutaneously with histamine every day for eight days. The percentages are plotted vertically, the days, horizontally. Note that during the first four days the mean percentage of non-granular leucocytes is lowered and from the fifth day on, is raised.

The changes in the eosinophile and neutrophile counts were not found to be statistically significant; the changes in the non-granular leucocyte counts, however, were suggestive (fig. 1). It can be seen that following the second and third injections the counts were lower and after the 5th and subsequent injections the counts were higher.

DISCUSSION

Following the successive injections of histamine the progressive hypertrophy and vacuolization of the medullary cells was interpreted as an indication of an increase in the secretory activity of these cells with the production of epinephrine and its release into the blood stream. Pekkarinen ('48) quotes figures which indicate that histamine induces an increase in circulating adrenaline. The extreme vacuolization seen in the majority of the medullary cells in the later stages of the series suggested exhaustion and hydropic degeneration. The finding (in the later stages) of mitotic figures in the medullary cells, which ordinarily are rare, suggested a compensatory hyperplasia in the adrenal medulla in some instances. If these interpretations are correct then histamine, or some by-product of its local action, or both, can act to stimulate the cells of the adrenal medulla. Moreover, the continued action of histamine may cause exhaustion of the medullary cells.

The progressive changes in the adrenal cortex following daily injections of histamine were interpreted as indicating that there was, in the first two days, an increase in secretory activity of the cells of the glomerular and transitional zones with some compensatory hyperplasia; that this change was followed, in the next two days, by an increase in secretory activity and hyperplasia of the cells of the zona fasciculata; and from the 5th day on, that there was failure of the secretory activity of the cells of the zona fasciculata associated with regression or atrophy of this zone. These interpretations therefore suggest that histamine (or its by-products) causes an increase in the production of adrenal cortical hormones either directly, or indirectly by means of stimulating epinephrine secretion in the medulla, which in turn may induce the secretion of ACTH. Farrell and McCann ('51) and McDermott et al. ('50) have deduced that epinephrine stimulates the production of ACTH by the anterior pituitary gland. It is difficult to explain the inhibition and regression of the

zona fasciculata of the adrenal cortex from the 5th to the 11th day of repeated injections of histamine. Three possibilities are suggested. (1) If the medullary cells become exhausted, undergo hydropic degeneration, and fail to secrete epinephrine, there may be no further stimulus for the production of ACTH. (2) Some antagonistic hormone may be called into play with the continued action of histamine, either from the glomerular and transitional zones of the cortex or from some other endocrine gland. (3) The thickening of the capillary basement membranes may be a factor in removing the cortical cells from the influence of trophic hormones in the blood. The fact that the glomerular and transitional zones of the cortex appeared to be active even when the zona fasciculata was reduced suggests that the cells of the outer zones are either independent of, or less sensitive to, the regulating mechanisms affecting the cells of the zona fasciculata.

It was hoped that daily differential blood counts on the larger series of animals might provide an index of the functional changes occurring in the adrenal glands. That there was no statistically significant variation in the percentages of eosinophiles is not surprising in view of the many factors involved. It has been shown by McDermott et al. ('50), that epinephrine causes a rise in circulating eosinophiles and that adrenocortical hormones cause a decrease in their number. Therefore, if histamine first caused secretion of epinephrine with a resulting eosinophilia and this was followed by cortical secretion with a resulting eosinopenia, then the two effects might be cancelled out in a large series of animals showing considerable individual variation in the rate and degree of reaction to histamine and hence an overlap in the responses.

The studies of Quittner et al. ('51) indicate that a single massive dose of cortisone depresses the circulating lymphocytes in the mouse. In this series, the correlation of the percentages of non-granular leucocytes and the appearances of the adrenal cortex (compare fig. 1 and figs. 2-5) supports the interpretation that there was first an increase and later

a failure of secretory activity in the zona fasciculata of the adrenal cortex.

CONCLUSIONS

These studies provide histological evidence that histamine is a "stressing" agent which stimulates the adrenal medulla to secrete epinephrine; this in turn, either directly, or more probably by stimulation of the anterior pituitary gland, causes an increase in adrenal cortical secretion. With repeated injections of histamine the adrenal medulla may become exhausted. This may be the basis for the failure of the adrenal cortex to respond to further injections of histamine.

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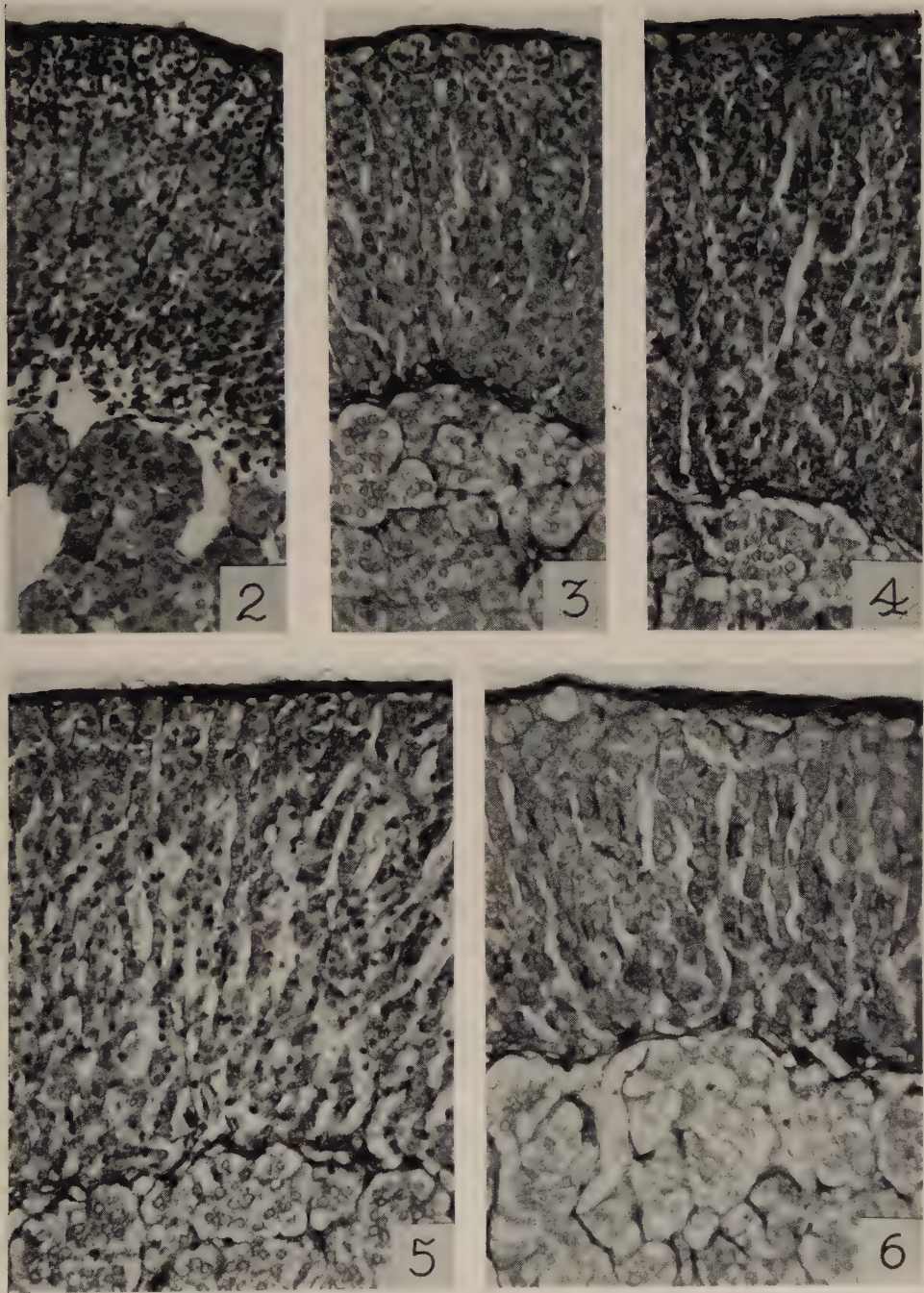
PLATES

PLATE 1

EXPLANATION OF FIGURES

Photomicrographs of 5μ sections of mouse adrenal glands fixed in formol-saline and stained with hematoxylin and the periodic acid-Schiff technic. $\times 400$.

- 2 Control mouse.
- 3 After two subcutaneous injections of histamine. Note the hypertrophy of the medullary cells and the staining of the reticular fibers at the cortico-medullary junction.
- 4 After three subcutaneous injections of histamine. Note the vacuolization of the medullary cells and the hypertrophy of the adrenal cortex (due to hyperplasia and hypertrophy of the cortical cells).
- 5 After 6 injections of histamine. Note the increase in the vacuolization of the medullary cells, the distended cortical capillaries and thinning of the cells in the zona fasciculata of the cortex.
- 6 After 7 injections of histamine. Note the hydropic degeneration of the medullary cells, the reduction in thickness of the cortex (due to the atrophy of zona fasciculata and reduction in size of zona reticularis), the thickening of the fibrous capsule, the prominent capillary basement membranes and the Schiff-positive cells at the cortico-medullary junction.



AXON TERMINALS IN THE NORMAL HUMAN CEREBRUM AND CEREBELLUM

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TEN FIGURES

INTRODUCTION

The fact that the nervous system is composed of discrete units or neurons in contact rather than continuity with each other, was first clearly demonstrated anatomically by Sharpey-Schafer (1878) in a paper in the *Philosophical Transactions*. In this little-known work, which he completed at the age of twenty-seven, Sharpey-Schafer described the discontinuous nerve elements in the umbrella of a jellyfish *Aurelia*, which he had found while visiting the Moray Firth in Scotland. Contrary to the then existing views, he concluded, as a result of his anatomical investigation, that each nerve cell in the umbrella of the little jellyfish carried messages to the next nerve cell in line by what he called "inductive action whether electrical or of some other kind."

This shrewd speculation was re-echoed ten years later in Spain where the unknown histological genius, Ramón y Cajal (1888), was publishing, in the first volume of his *Revista Trimestral*, a paper on "The Structure of the Nerve Centres of Birds." He scrutinized the microscopic structure of the retina stained with crude metallic methods, and independently came to the conclusion, that each nerve cell was in his words, "an absolutely autonomous local government." He saw the dendrites as gathering apparatuses which would collect im-

¹ Financed by a Federal Mental Health Grant.

pulses and funnel them through the cell body and down the unitary axon to its terminals where, by contact, the next nerve cell in the chain was excited "as a wire inducing a current." He cut clearly through the fog of supposed anastomosing end-filaments in the nervous system and thereby set the stage for a great anatomical and physiological generalization, "the theory of the dynamic polarization of the neurone." In this generalization Cajal was supported by Van Gehuchten (1891) in Belgium and by Sherrington (1897) in England.

Cajal soon showed that nerve fibres, when cut, degenerated peripherally in such a manner that silver salts were taken up rapidly and that their axonal terminals, the *boutons terminaux*, enlarged in size prior to eventual fragmentation. Hoff ('32), working at Oxford, used this method of staining for terminal degeneration in a number of studies, in an attempt to overcome the failure of the Marchi technique to demonstrate non-myelinated fibres, or the final termination of myelinated fibres. Over the ensuing years similar studies have been made by many eminent histologists, including De Castro ('30), Windle and Clark ('28), Lawrentjew ('34), Bartelmez and Hoerr ('33), Sheehan ('33), Nonidez ('37), Glees ('46), Bodian ('36), Barr ('39), Minckler ('40), Schadewald ('38), and Denber ('52), not only in the central nervous system but also in the autonomic system.

The work to be reported here stems from an effort to trace degenerating fibre tracts in human brains upon which the operation of frontal lobotomy has been performed. To establish, prior to such a study, the *normal* shape and distribution of the *boutons terminaux* in the human brain has been our first concern, however. The present paper deals only with the latter problem.

MATERIAL AND METHODS

In this work we have used a modification of the very simple and beautiful stain known as Rio-Hortega's Double Impregnation Method (Gibson, '50). It consists of the impregnation of 15 μ frozen sections of formalin-fixed human brain in a solu-

tion of silver nitrate, followed by silver carbonate and by gold toning. This well established method leads, in our experience, to clean impregnations with no overlay of deposited silver. In an attempt to establish the true picture of the distribution of axonal terminals upon nerve cells and their dendrites, we have used a photomosaic technique adapted from overlap aerial photography (Gibson, '53). Five human brains from individuals coming to autopsy for non-neurological causes were fixed in 10% formol and blocks cut as indicated. In addition, 18 biopsies, taken from patients at the time of lobotomy, were placed directly in 10% formol at the operating table and sectioned one month later.

RESULTS

The *boutons terminaux* in the human brain appear as single loops, 1 to 2μ in diameter. In favorable sections it can be shown that each loop is borne by a fine nerve fibre impinging upon a cell or its processes (fig. 1). In no case have we found protoplasmic bridges or filaments binding the boutons to the intracellular neurofibrils of the contacted cell. There was no discernible difference between the boutons in the post-mortem material and those in the biopsies of brain tissue.

With the present stain, as many as 11 contacts can be seen on that part of the surface of a cell in the normal human insular cortex, which appears in a single frozen section of 15μ thickness (fig. 2). In the cingulate area, one large dendritic process may carry a dozen *boutons terminaux* if, through good fortune, the frozen section is cut in the longitudinal plane (fig. 3). In the hippocampal gyrus, one can see in a single oil immersion field, using an $8\times$ ocular, as many as 70 bouton endings. The boutons vary somewhat in the size or length of loop, but the calibre of the fibre composing the ring-ending is remarkably constant.

It is notable that in the most superficial layer of the human cerebral cortex there are many long, horizontally disposed fibres which bear great numbers of swellings and occasionally

long loops in their longitudinal course. These are not to be mistaken for evidences of fibre degeneration. Rather, they may reflect the density of the small blood vessels and astrocytes deep to the pia which distort the fibres in transit.

With attention given to the temperature and pH of the silver carbonate bath it has become possible to reduce to a minimum the variations in staining which have for a long time plagued the student in this field. Briefly, a pH of 11.0 and a temperature of 40–45°C. in the silver carbonate bath have proven optimal for *boutons terminaux*. Sufficient length of fixation time in formol is another critical feature in the reproducibility of results in this work.

It is difficult to give, at this stage, any final figures on the true distribution of axonal endings in each of Brodmann's areas without extreme precautions. However, with the present staining method, we have found that *boutons terminaux* are relatively scarce in the visual cortex (fig. 4) and basal ganglia. They are *more* plentiful in the motor area (fig. 5), and the orbital surface of the frontal lobe. They are *most* plentiful in the hippocampal gyrus (fig. 6), in parts of the amygdaloid nucleus (fig. 7), in the cingulate gyrus (fig. 3), in the parolfactory area, and in the insula (fig. 2).

Axonal terminals have been stained in biopsy specimens of frontal cortex removed during lobotomy operations upon schizophrenics (figs. 8 and 9). Such terminals differ in no way from those found in post-mortem specimens of normal humans.

Fibre tract degeneration has been studied in brains of patients dying in the first two weeks following frontal lobotomy. This work will be the subject of a later report. Suffice it to say here that the rate of degeneration of axon terminals is considerably slower in the human cerebrum than in the spinal cord (Gibson, '37) and autonomic nervous system (Gibson, '40) of cats. The increased affinity for silver salts, and the swelling and eventual fragmentation of interrupted fibres and their endings, are found in the human cerebrum as in other areas of the nervous system.

Traditionally, axonal endings have been sought on the perikaryon rather than on the dendrites of nerve cells. In this investigation we have been impressed with the large proportion of *boutons terminaux* which appear on the dendrites, especially upon the apical dendrites of large cells in the third layer of the cortex, as in the cingulate gyrus (fig. 3). In the case of the Purkinje cells of the cerebellum, axon terminals in great numbers make contact with the widespread system of dendrites (fig. 10).

In addition to *boutons terminaux*, there are *boutons de passage* throughout the cerebrum and cerebellum, but in relatively small numbers. Chiefly in the superficial layer of the cerebral cortex are they to be found. No other types of termination have been encountered in this work.

DISCUSSION

It will be observed that the ring-like axonal terminals demonstrated in the human brain are similar in shape and size to those already stained in the spinal cord (Barr, '39; Gibson, '37) and in the autonomic ganglia (De Castro, '30; Lawrentjew, '34; Gibson, '40), differing chiefly in density of distribution.

It is not clear why it is more difficult to stain *boutons terminaux* in the cerebral cortex than in the spinal cord, for instance. This difference has been noted in block staining methods as well as in frozen section techniques.

It must be made clear that the present results are, in our opinion, indicative of only a fraction of what the cerebral cortex will yield once adequate stains are employed. Hypotheses based on negative results with silver stains are meaningless. It is for this reason that we reject the view advanced by Glees ('46) when he said that because *boutons terminaux* were difficult to demonstrate in the cerebral cortex we must assume that: "The synapse within the cortex is mainly represented by free terminals of the pericellular plexus." We have yet to see such "free terminals." With improved stains,

we confidently expect to find many more boutons. It is unlikely that the synaptic forms, standard for the rest of the nervous system, should be lacking specifically in the cerebral cortex.

If *boutons terminaux* are to be dismissed as artefacts, as some have suggested, then it must be agreed that the anterior horn cells of the spinal cord each have 1500 artefacts upon them (Barr, '39), and that the recent *in vivo* observations on axon terminals by Freedman ('52), in McCulloch's laboratory, are merely observations on living artefacts. Deformed though nervous tissue fixed in formalin may be, according to the excellent studies of Pomerat ('52), we nevertheless feel that the ring-like endings demonstrated with Rio-Hortega's stain are comparable to the living ring-like endings of the vagus nerve on the ganglion cells of the heart in the living frog, as observed by Freedman.

It was shown in a previous paper (Gibson, '40) that just as these boutons, when cut off from their mother cells in the sympathetic nervous system, fragment and disappear, so synaptic function in the superior cervical sympathetic ganglion ceases. Restoration of transmission returns with the demonstrable regeneration of bouton endings by the preganglionic fibres.

No attempt is made here to identify *boutons terminaux* with synapses. Such identification is felt, by some, to be unjustifiable. However, on embryological grounds (Harrison, '10), and on the basis of regeneration studies (De Castro, '30; Gibson, '40), it would seem logical to suggest that demonstrable axonal terminals constitute synaptic contacts between nerve cells. Beyond this it may be unwise to go, since other means of synapsis, as yet undemonstrated histologically, may be discovered.

The need for more specific stains for bouton endings has led to the introduction of further metallic methods by Glees ('46), Nauta ('54), Van Campenhout ('53), Weber ('48) and Denber ('52). However, specific cytochemical non-metallic stains would seem to offer the greatest rewards in this important field of basic neuroanatomy, and their refinement will

undoubtedly lead to more accurate work on normal and degenerating axonal terminations.

CONCLUSIONS

Ring-like *boutons terminaux* have been demonstrated as axonal endings in the human cerebellum and in the precen-tral cortex, parolfactory area, insular cortex, cingulate gyrus, visual cortex, hippocampal gyrus, orbital frontal cortex and amygdaloid nucleus.

The axonal endings are found on the perikaryon and den-drites of cortical nerve cells, especially on the apical dendrites of large pyramidal cells.

The distribution of *boutons terminaux* varies considerably for each area of the cortex, but in no part of the cerebrum or cerebellum does it approach the concentration of terminals upon the anterior horn cells of the spinal cord.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

- 1 Terminal fibre and a bouton terminal on a Purkinje cell of the cerebellum.
× 3300.
- 2 A cell from the insula showing eleven bouton endings surrounding the cell.
× 3300.

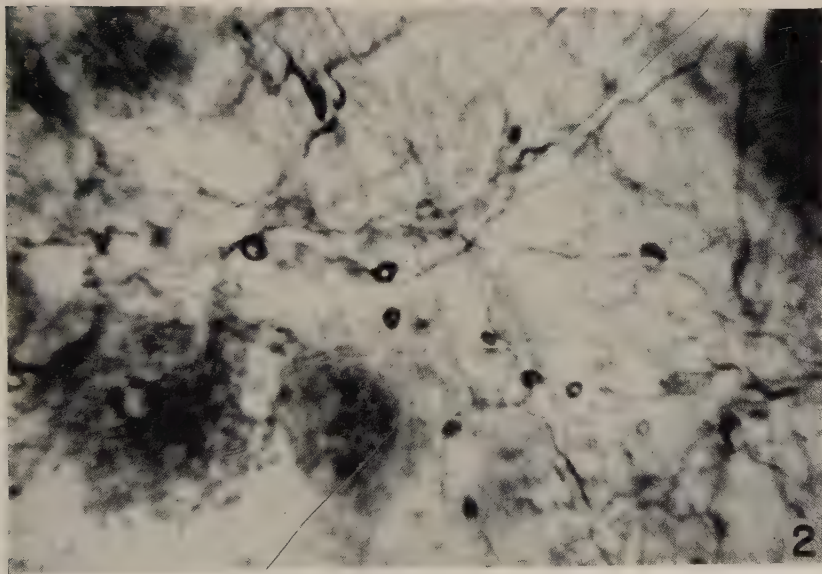
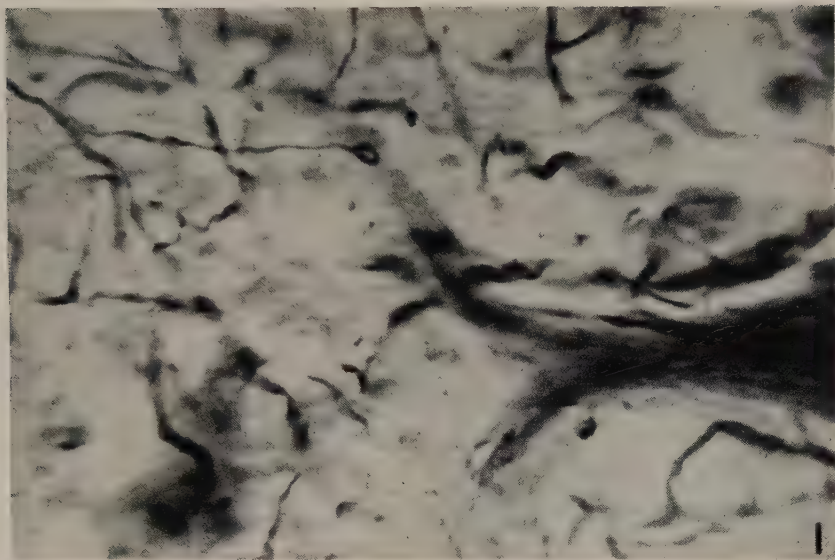


PLATE 2

EXPLANATION OF FIGURES

- 3 *Boutons terminaux* along one dendritic process in the cingulate cortex.
× 1200.
- 4 Terminations in the visual cortex. × 1500.
- 5 Motor cortex, showing ring-like endings. × 1500.

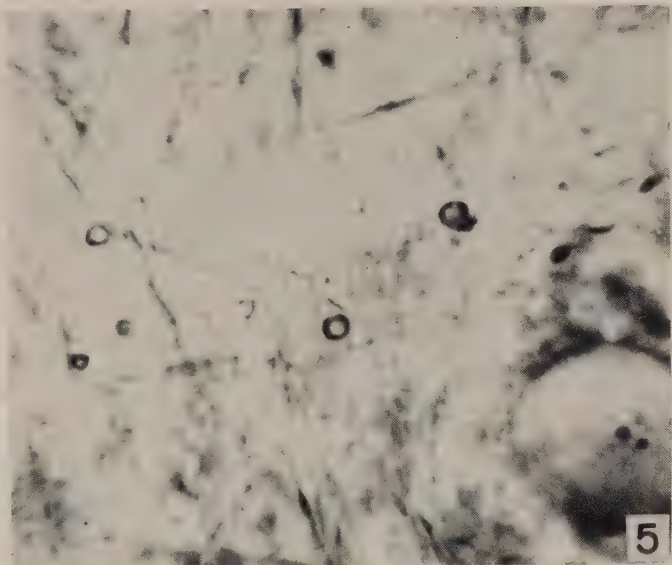
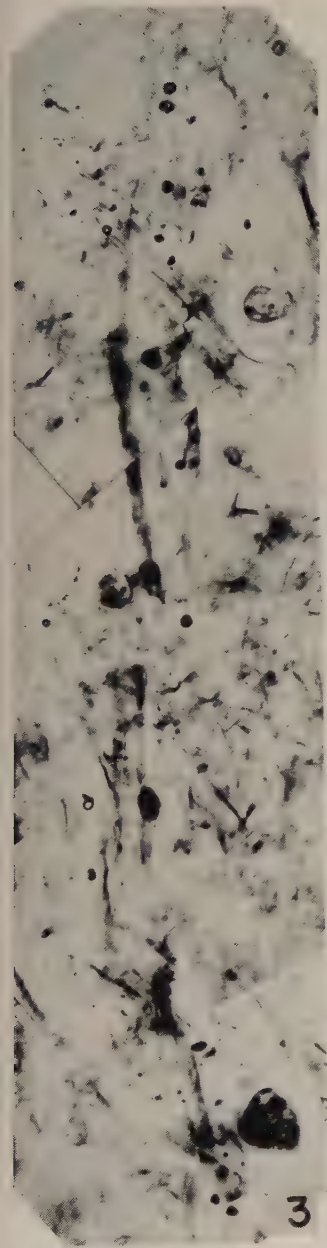


PLATE 3

EXPLANATION OF FIGURES

- 6 *Bouton terminaux* in the hippocampal gyrus.
7 *Bouton terminaux* in the amygdaloid nucleus.

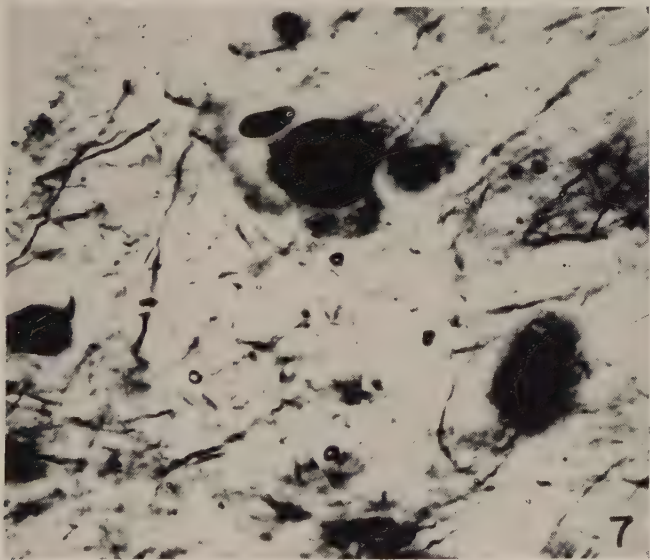
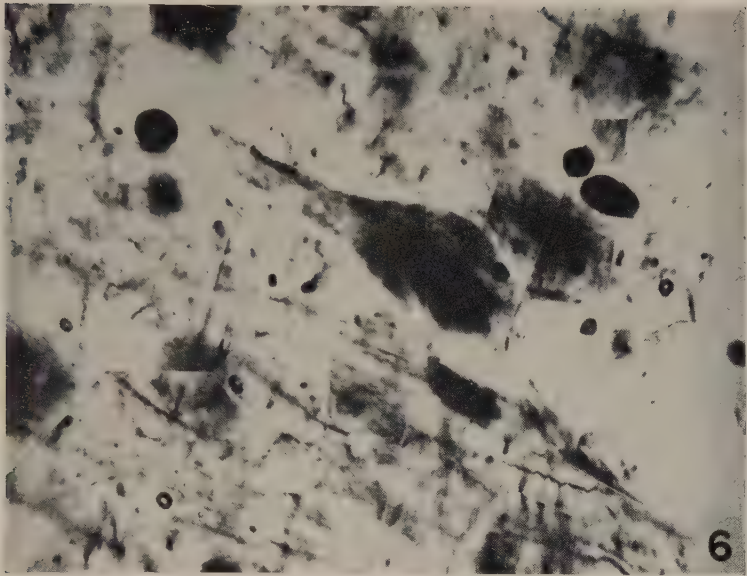


PLATE 4

EXPLANATION OF FIGURES

- 8 Frontal cortex biopsy from schizophrenic brain.
- 9 Frontal cortex biopsy from schizophrenic brain.

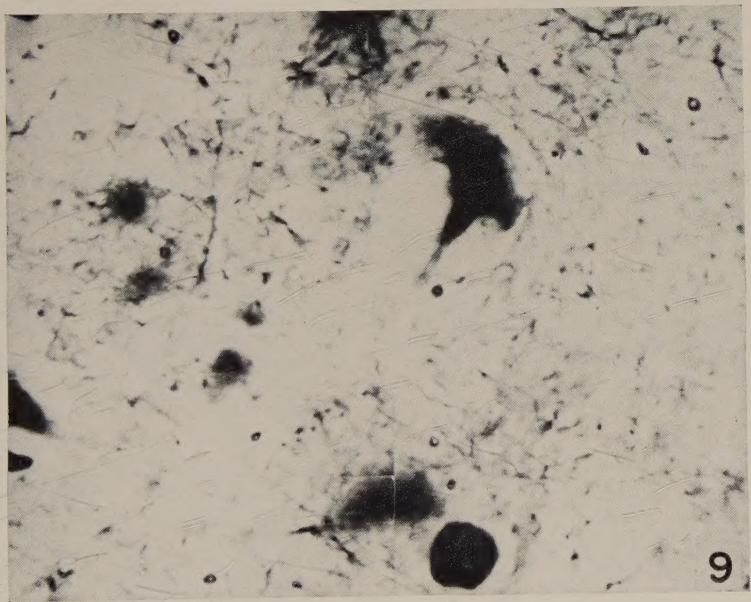
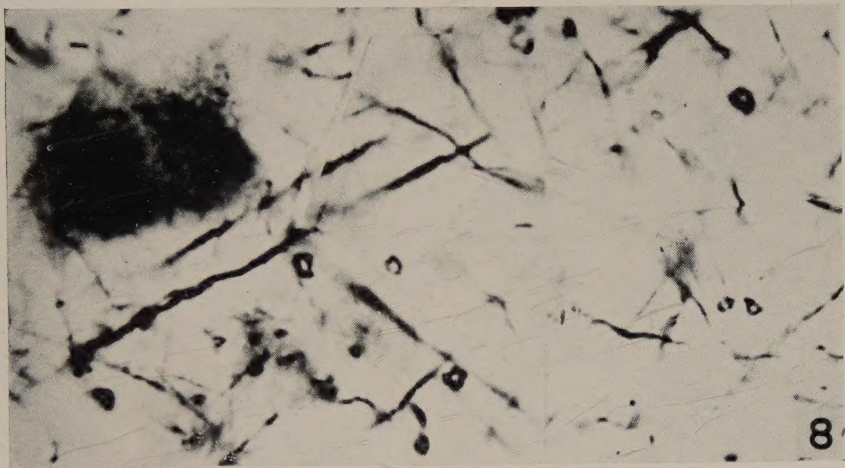


PLATE 5

EXPLANATION OF FIGURE

10 Axon terminals on the dendrites of Purkinje cells of the cerebellum.

